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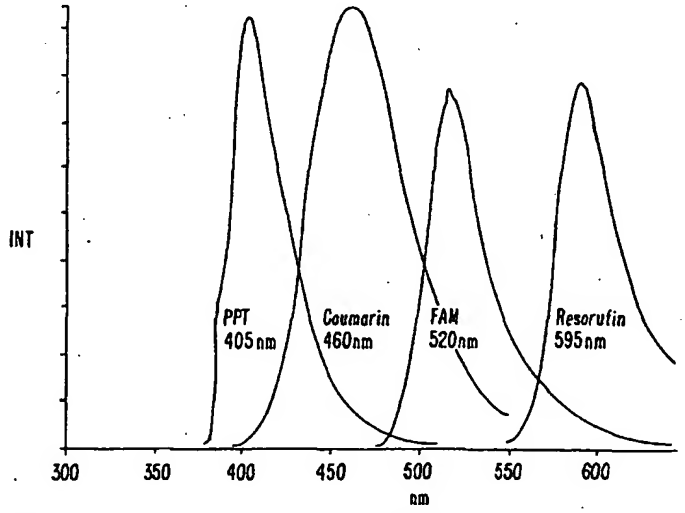

(57) Abstract: Oligonucleotide-fluorophore-quencher conjugates wherein the fluorophore moiety has emission wavelengths in the range of about (300) to about (800) nm, and or where the quencher includes a substituted 4-(phenyldiazenyl)phenylamine structure provide improved signal to noise ratios and other advantageous characteristics in hybridization and related assays. The oligonucleotide-fluorophore-quencher conjugates can be synthesized by utilizing novel phosphoramidite reagents that incorporate the quencher moiety based on the substituted 4-(phenyldiazenyl)phenylamine structure, and or novel phosphoramidite reagents that incorporate a fluorophore moiety based on the substituted coumarin, substituted 7-hydroxy-3H-phenoxazin-3-one, or substituted 5,10-dihydro-10-[phenyl]pyrido[2,3-d;6,5-d']dipyrimidine-2,4,6,8-(1H, 3H, 7H, 9H, 10H)-tetrone structure. Oligonucleotide-fluorophore-quencher-minor groove binder conjugates including a pyrrolo[4,5-e]indolin-7-yl}carbonyl)pyrrolo[4,5-e]indolin-7-yl]carbonyl}pyrrolo[4,5-e]indoline-7-carboxylate (DPI_3) moiety as the minor groove binder and the substituted 4-(phenyldiazenyl)phenylamine moiety as the quencher, were synthesized and have substantially improved hybridization and signal to noise ratio properties.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

FLUORESCENT QUENCHING DETECTION REAGENTS AND METHODS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to oligonucleotide-quencher-fluorescent-dye conjugates having improved characteristics, and to reagents suitable for incorporating novel quencher and fluorescent dye moieties into oligonucleotides. The invention also relates to the use of oligonucleotide-quencher-fluorescent-dye conjugates in detection methods for nucleic acid targets.

2. Brief Description of Related Art

Synthetic oligonucleotides have been used for years as sequence specific probes for complementary DNA and RNA targets. These methods have broad application in forensics, molecular biology and medical diagnostics since they allow the identification and quantitation of specific nucleic acid targets. Early uses of DNA probes relied on radioactivity (typically ^{32}P) as the label, while recent methods use reporter molecules which include chemiluminescent and fluorescent groups. Improved instrumentation has allowed the sensitivity of these spectroscopic methods to approach or surpass the radiolabeled methods. Recently developed detection methods employ the process of fluorescence resonance energy transfer (FRET) for the detection of probe hybridization rather than direct detection of fluorescence intensity. In this type of assay, FRET occurs between a donor fluorophore (reporter) and an acceptor molecule (quencher) when the absorption spectrum of the quencher molecule overlaps with the emission spectrum of the donor fluorophore and the two molecules are in close proximity. The excited-state energy of the donor fluorophore is transferred to the neighboring acceptor by a resonance dipole-induced dipole interaction,

which results in quenching of the donor fluorescence. If the acceptor molecule is a fluorophore, its fluorescence may sometimes be increased. The efficiency of the energy transfer between the donor and acceptor molecules is highly dependent on distance between the molecules. Equations describing this relationship are known. The Forster distance (R_o) is described as the distance between the donor and acceptor molecules where the energy transfer is 50% efficient. Other mechanisms of fluorescence quenching are also known, such as, collisional and charge transfer quenching.

Typically detection methods based on FRET are designed in such a way that the donor fluorophore and acceptor molecules are in close proximity so that quenching of the donor fluorescence is efficient. During the assay, the donor and acceptor molecules are separated such that fluorescence occurs. FRET-based detection assays have been developed in the fields of nucleic acid hybridization and enzymology. Several forms of the FRET hybridization assays are reviewed (Nonisotopic DNA Probe Techniques, Academic Press, Inc., San Diego 1992, pp. 311-352).

Since its discovery, the polymerase chain reaction (PCR) has revolutionized molecular biology. This technique allows amplification of specific DNA sequences, thus allowing DNA probe assays to be executed from a single DNA target copy. PCR-based diagnostic assays have initially not been used routinely, in part due to problems with sample handling and possible contamination with non-source DNA. Recently, new homogeneous fluorescent-based DNA assays have been described which can detect the progress of PCR as it occurs ("real-time" PCR detection) using spectrofluorometric temperature cyclers. Two popular assay formats use DNA probes which become fluorescent as DNA amplification occurs (fluorogenic probes).

The first format for "real-time" PCR uses DNA probes known as "molecular beacons" (*Tyagi et al.*, Nat. Biotech., 16: 49-53 (1998)). Molecular beacons have a hairpin structure wherein the quencher dye and

reporter dye are in intimate contact with each other at the end of the stem of the hairpin. Upon hybridization with a complementary sequence, the loop of the hairpin structure becomes double stranded and forces the quencher and reporter dye apart, thus generating a fluorescent signal. *Tyagi et al.* reported use of the non-fluorescent quencher dyes including the dabcyl (4-{[4-(dimethylamino)phenyl]diazenyl}benzoyl moiety, absorbance max=453 nm) used in combination with fluorescent reporter dyes of widely varying emission wavelength (475-615 nm). At the time this was surprising since FRET requires significant overlap of the absorption spectrum of the quencher and of the emission spectrum of the reporter. In case of a dabcyl moiety containing (hereinafter "dabcyl") quencher and some fluorescent dyes, the spectral overlap was extremely low, yet quenching efficiency was high. Therefore it was proposed that the mechanism of quenching for the hairpin form of the beacons was not FRET, but collisional quenching. In fact, the UV spectra of the quencher changes in the hairpin form of the beacon, providing evidence of the molecular contact and thus of collisional quenching. A related detection method uses hairpin primers as the fluorogenic probe (Nazarenko et al., Nucl. Acid Res., 25: 2516-2521 (1997)).

The second format for "real-time" PCR uses DNA probes which are referred to as "5'-nuclease probes" (*Lee et al.*, Nucl. Acid Res., 21: 3761-3766 (1993)). These fluorogenic probes are typically prepared with the quencher at the 3' terminus of a single DNA strand and the fluorophore at the 5' terminus. During each PCR cycle, the 5'-nuclease activity of *Taq* DNA polymerase cleaves the DNA strand, thereby separating the fluorophore from the quencher and releasing the fluorescent signal. The 5'-nuclease assay requires that the probe be hybridized to the template strand during the primer extension step (60-65°C). They also disclose the simultaneous "real-time" detection of more than one polynucleotide sequence in the same assay, using more than one fluorophore/quencher pair. The 5'-nuclease PCR assay is depicted in **Figure 1**.

Initially it was believed that 5'-nuclease probes had to be prepared with the quencher (usually tetramethylrhodamine (TAMRA)) positioned at an internal nucleotide in close proximity to the 5'-fluorophore (usually fluorescein (FAM) or tetrachlorofluorescein (TET)) to get efficient FRET. Later it was found that this is not necessary, and the quencher and the fluorophore can be located at the 3' and 5' end of the ODN, respectively. It has been proposed that the random coil structures formed by these fluorogenic probes in solution allow a 3'-quencher dye to pass within the Forster radius of the 5'-fluorophore during the excited state of the molecule.

A number of donor/acceptor pairs have previously been described, important to the present invention is dabcyl that is used for instance as a quencher of dansyl sulphonamide in chemosensors (*Rothman & Still* (1999) Med. Chem. Lett. 22, 509 - 512).

Surprisingly, there have been no published reports on the use of dabcyl in 5'-nuclease probes or other FRET probes that use long wavelength fluorophores. As mentioned above, dabcyl was used in the beacon-type probes but this is a different quenching mechanism wherein the dabcyl and fluorophore are in intimate contact (collisional quenching). Dabcyl was used in fluorogenic peptides as a quencher for the fluorophore EDANS (5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid) which emits at short (490 nm , blue) wavelength (*Matayoshi et al.* Science 247: 954-958 (1990)). EDANS also has a lower extinction coefficient than dabcyl so it is not surprising that fluorescent quenching was efficient. It was found for the first time in the present invention that dabcyl can be used to quench fluorescein in a FRET type mechanism.

In addition to the 5'-nuclease PCR assay, other formats have been developed that use the FRET mechanism. For example, single-stranded signal primers have been modified by linkage to two dyes to form a donor/acceptor dye pair in such a way that fluorescence of the first dye is quenched by the second dye. This signal primer contains a restriction site

(United States Patent No. 5,846,726) that allows the appropriate restriction enzyme to nick the primer when hybridized to a target. This cleavage separates the two dyes and a change in fluorescence is observed due to a decrease in quenching. Non- nucleotide linking reagents to couple oligonucleotides to ligands have also been described (United States Patent No. 5,696,251).

FRET systems also have applications in enzymology. Protease cleavable substrates have been developed where donor/acceptor dye pairs are designed into the substrate. Enzymatic cleavage of the substrate separates the donor/acceptor pair and a change in fluorescence is observed due to a decrease in quenching. Cleavable donor/acceptor substrates have been developed for chymotrypsin (*Li et al.* Bioconj. Chem., 10: 241-245 (1999)), aminopeptidase P (*Hawthorne et al.*, Anal. Biochem., 253: 13-17 (1997)), stromelysin (*Bickett et al.*, Ann. N. Y. Acad. Sci., 732: 351-355 (1994)) and leukotriene D_4 hydrolase (*White et al.*, Anal. Biochem., 268: 245-251 (1999)). A chemosensor was described where binding of the ligand separates the donor/acceptor pair (*Rothman et al.* Biorg. Med. Chem. Lett., 9: 509-512 (1999)).

In United States Patent No. 5,801,155 it was disclosed that oligonucleotides (ODNs) having a covalently attached minor groove binder (MGB) are more sequence specific for their complementary targets than unmodified oligonucleotides. In addition the MGB-ODNs show substantial increase in hybrid stability with complementary DNA target strands when compared to unmodified oligonucleotides, allowing hybridization with shorter oligonucleotides.

Reagents for fluorescent labeling of oligonucleotides are critical for efficient application of the FRET assays described above. Other applications such as DNA micro arrays also use fluorescently labeled DNA probes or primers, and there is a need for improved reagents which facilitate synthesis of fluorescent DNA. In general, phosphoramidite reagents and solid supports

are widely used on ODN synthesis. However, in the state of the art there are not many commercially available phosphoramidite reagents for introducing fluorescent groups into ODNs.

Linker groups to attach different ligand groups to ODNs play an important role in the synthesis of oligonucleotide conjugates. A method for the synthesis of 3'-aminohexyl-tailed oligonucleotides (*Petrie et al.*, Bioconj. Chem., 3: 85-87 (1992)), the use of a trifunctional trans-4-hydroxy-L-prolinol group (*Reed et al.*, Bioconjug. Chem., 2: 217-225 (1991)), diglycolic acid (*Pon et al.*, Nucl. Acids. Res., 25: 3629-3635 (1997)), 1,3-diol reagents (US 5,942,610 and US 5,451,463) and the non-nucleotide trifunctional reagent (US 5,696,251) have been reported.

Resorufin and coumarin derivatives have been extensively used as enzyme substrates to differentiate isozymes of cytochrome P450 (*Haugland et al.*, Handbook of Fluorescent Probes and Research Chemicals, Six Edition, Eugene, OR. pp. 235-236. 1996.). Reactive resorufin analogs have been disclosed in United States Patent No. 5,304,645. Activated esters of coumarin derivatives are known in the art (*Hirshberg et al.*, Biochem., 37: 10391-5 (1998)). Coumarin-labeled dUTP incorporated in probes were used for *in situ* hybridizations (*Wiegant et al.*, Cytogenet. Cell Genet., 63: 73-76 (1993)). Phosphoramidites to introduce labels into oligonucleotides have been described in United States Patent Nos. 5,328,824 and 5,824,796.

Many current hybridization applications, require more than one reporter molecule. In addition although reporter fluorophores are available to be used in reporter/quencher pairs, most suffer from having some undesirable characteristic, mixtures difficult to separate, positively charged, difficult to synthesize, unstable during oligonucleotide synthesis or having overlapping emission wavelengths with other desirable reporters. The present invention provides reagents for oligonucleotide probes that address these unfavorable characteristics and overcome some or all of the difficulties.

SUMMARY OF THE INVENTION

The present invention provides quencher molecules based on the 4-[4-nitrophenyl)diazinyl]phenylamine and/or the 4-[4-nitrophenyl)diazinyl]-naphthylamine structure. The quencher molecules have improved UV spectral overlap not only with commonly used fluorescent reporter groups that emit short wavelength range (about 400 to 500 nm), but have extended the range to the mid (525 nm = green) to long (670 nm = red) wavelengths. The quencher chromophores of the present invention are non-fluorescent, easily incorporated into DNA synthesis reagents, stable during automated DNA synthesis and during storage and compatible with no adverse effects on hybridization properties. Moreover, improved signal to noise ratios are observed with the fluorescent reporter dyes over a more extended wavelength range. Thus the present invention offers considerable advantages over the use of dabcyl (Nazerenko et al, Nucl. Acids Res., 25; 2516-21 (1997)) as a quenching dye, as used in the prior art.

In accordance with one aspect of the present invention the quenchers based on the 4-[4-nitrophenyl)diazinyl]phenylamine (and/or the 4-[4-nitrophenyl)diazinyl]naphthylamine structure) are modified with linker structures that allow their easy incorporation into fluorogenic DNA probes during automated DNA synthesis. The invention includes synthesis of phosphoramidites derived from the novel quencher molecules for incorporation of the quencher moieties into oligonucleotides during automated synthesis, and also synthesis of reagents derived from the novel quencher molecules for post solid-phase support attachment to amino-tailed oligonucleotides. In a related aspect, the novel quencher molecules are introduced into oligonucleotides using pyrazolo-[5,4-d]pyrimidines and pyrimidines phosphoramidites containing the quenchers attached at the 3'- and 5'-positions, respectively.

In accordance with another aspect of the invention, three different

fluorescent reagent types that are compatible with DNA synthesis are synthesized or selected and converted into phosphoramidite reagents suitable for incorporation onto ODNs. Specifically, violet fluorescent dyes based on the 10-phenyl-1,3,5,7,9,10-hexahydropyrimidino[5',4'-5,6]pyridino[2,3-d]pyrimidine-2,4,6,8-tetraone (PPT) structure, red fluorescent dyes based on 7-hydroxyphenoxazin-3-one (resorufin) and blue fluorescent dyes based on the structure of coumarin are incorporated into phosphoramidite reagents. These fluorescent dyes have excellent properties for multicolor fluorescent analysis in combination with other dyes (eg. fluorescein). These reagents are valuable for a variety of analytical methods that use either direct detection of fluorescence or FRET detection formats. In a related aspect of the invention the PPT-, coumarin- and resorufin-based fluorophores (fluorescent dyes) are converted into novel reagents suitable for "post-oligonucleotide-synthesis" covalent attachment at the 5'-end of ODNs. In another aspect, the new fluorescent dyes are incorporated into oligonucleotides using pyrazolo-[5,4-d]pyrimidines and pyrimidines phosphoramidites which contain the fluorophores attached at the 3- and 5-positions, respectively.

In accordance with still another aspect of the invention, ODNs covalently linked with the novel quencher structures of the invention, paired with a covalently attached fluorescent moieties, are prepared. The resulting FL-ODN-Q conjugate may also include a minor groove binder (MGB) that improves the binding and discrimination characteristics of the resulting FL-ODN-Q-MGB conjugate in diagnostic assays, particularly in the TaqMan PCR assay of single nucleotide polymorphism (and the like) where allele-specific discrimination not only requires probes with different fluorescent reporter molecules but efficient quenchers. The quenchers used in accordance with the invention in the FL-ODN-Q-MGB conjugates provide broad quenching wavelength range, and certain novel reporter labeling reagents in accordance with the invention have distinctive emission wavelengths for improved multicolor analysis.

In one application of the principles summarized above, fluorogenic probes are prepared using a universal "3'-hexanol" solid support (available in accordance with *Gamper et al.* Nucleic Acids Res., 21: 145-150 (1993) expressly incorporated herein by reference), where a quencher phosphoramidite of the invention is added at the first coupling step (3'-end) of the ODN sequence and a fluorophore (FL) was attached at the final coupling step, yielding 5'-FL-ODN-Q-hexanol conjugate probes.

As other applications of the invention, methods for synthesizing and attaching the novel quenchers to ODN-fluorophore conjugates with and without a 3'-minor groove binder (MGB) are disclosed. These methods utilize synthetic solid supports for automated oligonucleotide synthesis with cleavable linkers.

In another application, a fluorogenic oligonucleotide probe is prepared from a MGB modified solid support substantially in accordance with the procedure of *Lukhtanov et al.* Bioconjugate Chem., 7: 564-567 (1996), where a quencher- phosphoramidite of the invention is added at the first coupling step to the MGB, and a fluorophore (FL) is attached at the final coupling step to the ODN, to yield 5'-FL-ODN-Q-MGB conjugate probe.

Additional application of the methods and compositions of the present invention are in micro-arrays in nucleic acid-based diagnostic assays which recently have become important in many fields, such as the medical sciences, forensics, agriculture and water quality control. Other related application of the methods and compositions of the present invention are in procedures using arrays of oligonucleotides, such as the array-based analysis of gene expression (*Eisen*, Methods of Enzym., 303: 179-205 (1999)). In these procedures, an ordered array of oligonucleotides or DNAs that correspond to all, or a large fraction of the genes in many organism is used as a platform for hybridization. Microarray-based methods are used in assays to measure the relative representation of expressed RNA species. The quantitation of differences in abundance of each RNA species is achieved by directly

comparing two samples by labeling them with spectrally distinct fluorescent dyes and mixing the two probes for simultaneous hybridization to one array.

To the extent the application of the compositions and methods of present invention relates to the detection of nucleic acids, it includes but is not limited to methods where FRET is involved, such as 5'-nuclease, universal energy transfer primers or beacon assays. These methods are usually directed to, but are not limited to the detection of PCR-generated nucleic acid sequences. Some of these methods involve simultaneous detection of more than one nucleic acid sequence in the same assay. Similarly, the invention relates to methods where FRET is involved in the detection of protein concentration or enzyme activity.

Still other applications of the invention relate to the labeling with luminescent PPT- , coumarin- and resorufin-based dyes of nucleic acids, proteins and other materials including, drugs, toxins, cells, microbial materials, particles, glass or polymeric surfaces and the like, at a reactive group such as an amino, hydroxyl or sulfhydryl group. The present invention may be used in single- and two-step labeling processes. In the two-step labeling process, a primary component, such as an oligonucleotide is labeled with the reagent capable of introducing the novel fluorophore PPT- , coumarin- and resorufin-based dyes, by reaction with a reactive group of the ODN (such as an amine, hydroxyl, carboxyl, aldehyde or sulfhydryl group) and the label is used to probe for a secondary component, such as an oligonucleotide target.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of real-time 5'-nuclease PCR assay.

Figure 2 is a graph showing the UV spectra of Dabcyl- and Red13 dye-modified DNA probes.

Figure 3 is a graph showing the performance of fluorogenic MGB probes in a "real-time" PCR assay.

Figure 4 is a graph showing the fluorescent spectra of violet, FAM and resorufin dye containing DNA probes .

DETAILED DESCRIPTION OF INVENTION

QUENCHER REAGENTS FOR OLIGONUCLEOTIDE SYNTHESIS

Two types of reagents for introducing the substituted 4-(phenyldiazenyl)phenylamine quencher moieties into oligonucleotides using an automated DNA synthesizer are exemplified in the disclosure below. Here and in the reaction schemes the abbreviations MGB, FL, Q, CPG and ODN stand for "minor groove binder", "fluorescent or fluorophor", "quencher" "controlled pore glass" and "oligonucleotide" moieties or molecules, respectively, and in a manner which is apparent from context.

Dimethoxytrityl Protected Quencher Phosphoramidites

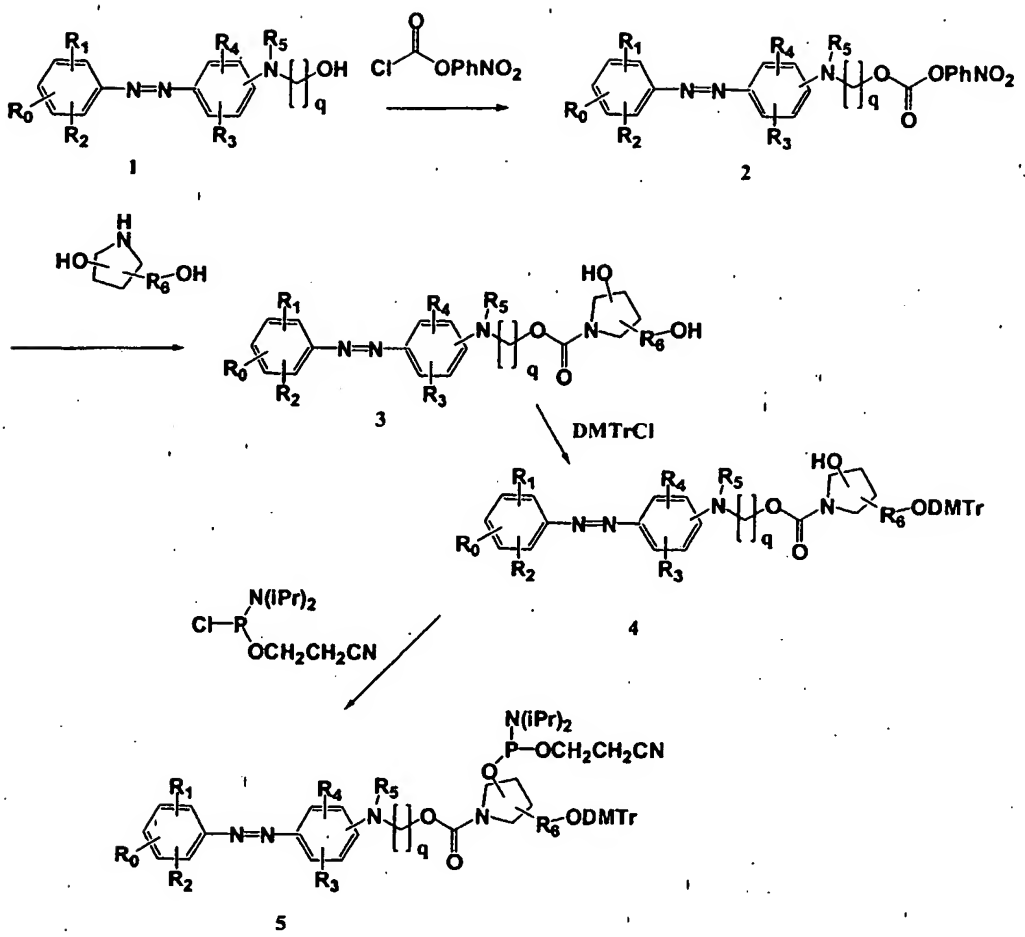
The first type of reagents disclosed herein are phosphoramidites that bear the quencher molecule (Q) as well as a dimethoxytrityl (DMTr) (methoxytrityl, trityl or the like acid labile blocking group) protected primary alcohol that provides an attachment point for the growing oligodeoxynucleotide (ODN) chain during subsequent oligonucleotide synthesis. Examples of these reagents are depicted in **Formulas 1, 2,** and **3,** and in **Reaction Schemes 1** and **2.**

In **Reaction Scheme 1** the starting compound is a substituted 4-(phenyldiazenyl)phenylamine **1** that has a primary hydroxyl group. Such starting material is commercially available or can be synthesized in accordance with methods known in the art, applying routine skill available to the practicing organic chemist. For example 4-nitrobenzendiazonium salt is reacted with 2-(2-chloroanilino)ethanol to yield 2-[2-chloro-4-(4-nitrophenylazo)anilino]ethanol in accordance with the teachings of United

States Patent No. 2,264,303. 2-[2-chloro-4-(4-nitrophenylazo)anilino]ethanol is within the scope of compound **1** as depicted in **Reaction Scheme 1**. The specification of United States Patent No. 2,264,303 is expressly incorporated herein by reference.

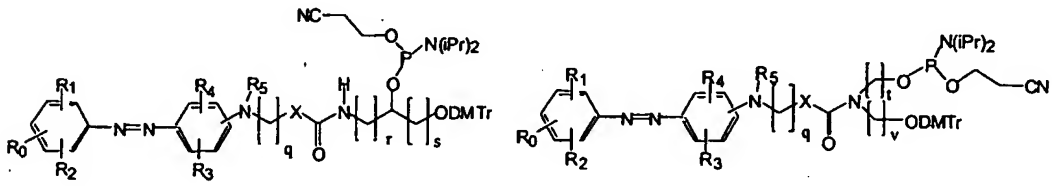
Other examples of commercially available starting materials (or of their precursors) are: 2-(ethyl{4-[(4-nitrophenyl)diazenyl]phenyl}amino)-ethan-1-ol and 2-(ethyl{4-[(2-methoxy-4-nitrophenyl)diazenyl]phenyl}-amino)ethan-1-ol.

As is shown in **Reaction Scheme 1,** compound **1** is reacted with p-nitrophenylchloroformate to yield the carbonate **2**. Reaction of **2** with substituted pyrrolidinediols yields a diol intermediate **3**. The pyrrolidinediol is a trifunctional reagent that has an amino, a primary and a secondary hydroxyl group. An example of a pyrrolidinediol as well as examples of other trifunctional reagents having an amino, primary and a secondary hydroxyl group, are described in United States Patent No. 5,512,667 the specification of which is incorporated herein by reference. The diol **3** is reacted first with dimethoxytrityl chloride (DMTrCl) to block the primary hydroxyl group of the trifunctional reagent and yield intermediate **4**. The intermediate **4**, still having a free secondary hydroxyl group in the trifunctional reagent, is then reacted with 2-cyanoethyl diisopropylchlorophosphoramidite to give the dimethoxytrityl protected phosphoramidite reagent **5**. In the compounds shown in **Reaction Scheme 1** the symbols are defined as follows. R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,$-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$ where n= 0 to 5, $-NO_2$, $-SO_3$, $-N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN; R_5= -H or $-(CH_2)_{n''}CH_3$ where n"= 0 to 5; R_6=$-(CH_2)_{n^*}$ where n*= 1 to 5 and q= 1 to 20. The dimethoxytrityl protected phosphoramidite reagent **5** is suitable for attachment to oligonucleotides in steps otherwise known in routine ODN synthesis.



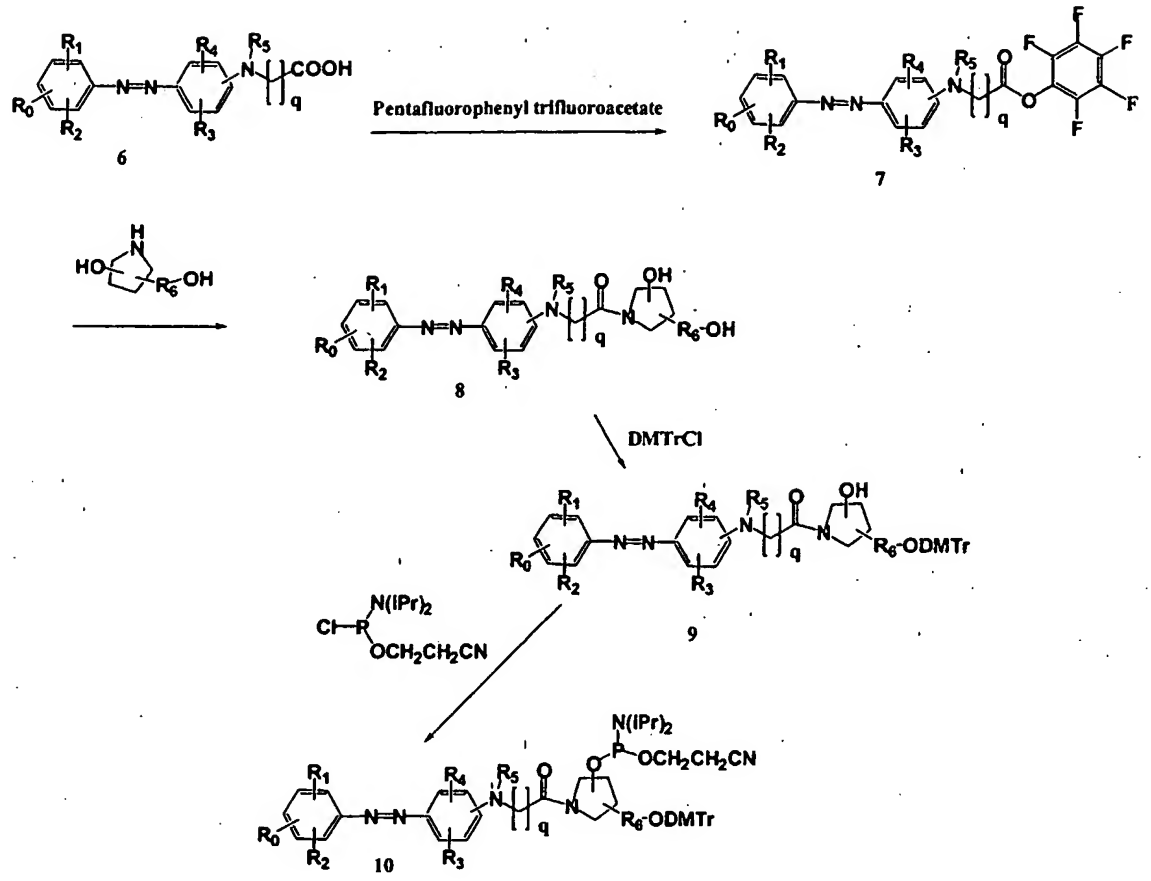
REACTION SCHEME 1

In other embodiments, using the reactions described in **Reaction Scheme 1**, (or only such modifications which are within the skill of the practicing organic chemist) starting with other trifunctional reagents having an amino and two hydroxyl groups, the phosphoramidites of **Formula 1** and **Formula 2** are synthesized, where q, R_0, R_1, R_2, R_3, R_4 and R_5, are defined as above; r and s independently are 1 to 20; X is –O- or -CH_2-; t and v independently are 1 to 20.



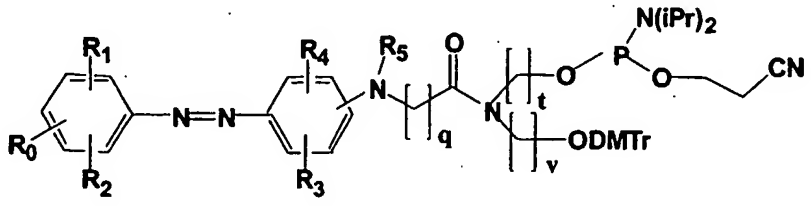
Formula 1 Formula 2

Reaction Scheme 2 discloses the synthesis of another exemplary phosphoramidite reagent **10** bearing the substituted 4-(phenyldiazenyl)-phenylamine quencher moiety and including the trifunctional pyrrolidinediol moiety. In this synthetic scheme the starting material is a substituted 4-(phenyldiazenyl)phenylamine compound **6** that has a free carboxyl group. Compound **6** (commercially available or made in accordance with the chemical literature within the skill of the practicing organic chemist) is reacted with pentafluorophenyl trifluoroacetate to make an active ester **7**, which is thereafter reacted to couple the substituted 4-(phenyldiazenyl)-phenylamine moiety to the ring nitrogen of a pyrrolidinediol moiety having a free primary and a free secondary hydroxyl group, yielding compound **8**. Treatment of **8** with DMTrCl followed by reaction with 2-cyanoethyl diisopropylchlorophosphoramidite gives the dimethoxytrityl protected phosphoramidite reagent **10**. In **Reaction Scheme 2** the symbols are defined the same as in **Reaction Scheme 1**.



REACTION SCHEME 2

In still another example, using the reactions described in **Reaction Scheme 2**, starting with a substituted 4-(phenyldiazenyl)phenylamine (compound **6**) and using a non-cyclic trifunctional reagent (having an amino and two hydroxyl functions) instead of the pyrrolidinediol shown in **Scheme 2**, the dimethoxytrityl protected phosphoramidite of **Formula 3** is synthesized, where q, R_0, R_1, R_2, R_3, R_4, R_5, and R_6 are defined as above and t and v independently are 1 to 20.

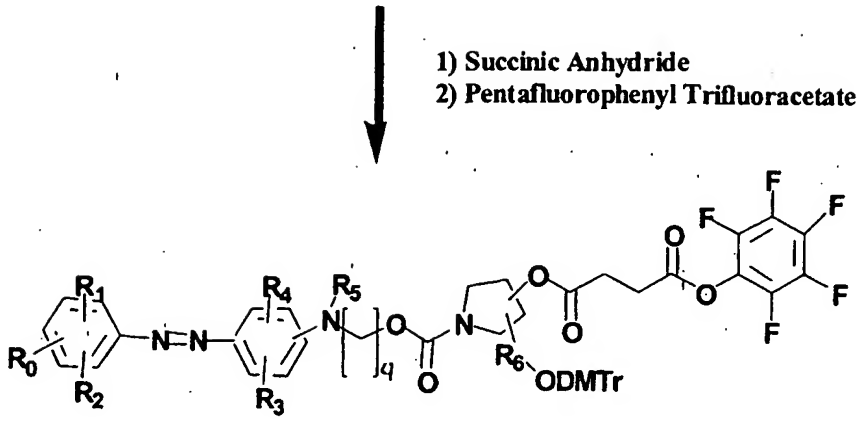


Formula 3

Quenchers Attached to Solid Support through (or similarly) Protected Linker, Suitable for ODN Synthesis

A second class of compounds or reagents suitable for introducing the quencher molecules into ODNs constitute a composition that has a solid support of the type used for ODN synthesis (for example controlled pore glass (CPG)), and linker attaching the quencher to the solid support. The linker has a hydroxyl function that is protected, usually by a dimethoxytrityl group which is removed during the synthesis when the first nucleotide is attached to the linker. Generally speaking the same quencher/linker intermediates described above in **Reaction Scheme 1** can also be used to prepare these reagents (CPG beads) having the exemplary structure **12**, shown in **Reaction Scheme 3**.

4



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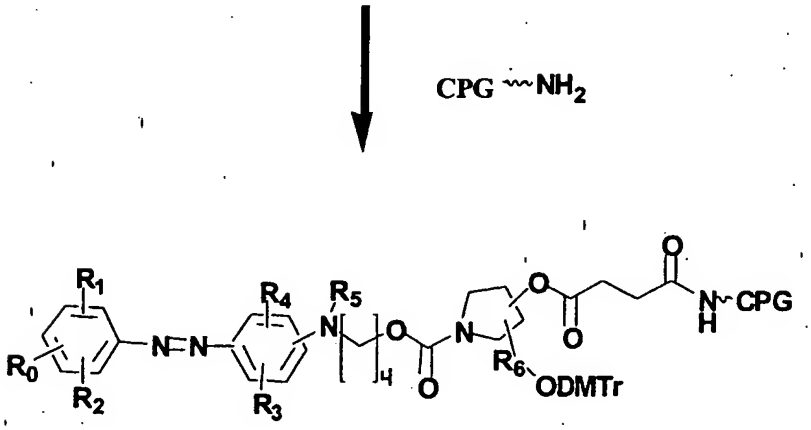


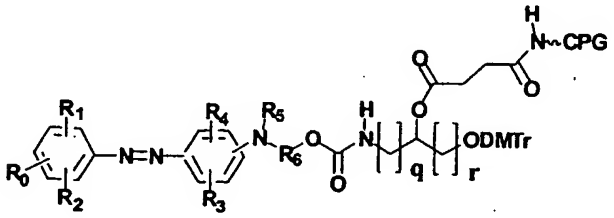
12

REACTION SCHEME 3

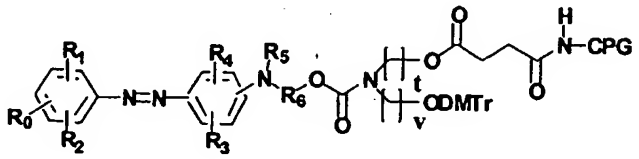
In accordance with this scheme, the secondary hydroxyl group of the intermediate **4** (shown in **Scheme 1**) is reacted with succinic anhydride, and thereafter pentafluorophenyl trifluoroacetate to provide the active ester **11**. The active ester **11** is then reacted with the free amino group attached to the solid support (CPG bead) to provide the modified solid support **12**. Whereas the exemplary modified solid support **12** includes the "trifunctional linker"

derived from pyrrolidine diol, it will be readily understood by those skilled in art that analogous modified solid supports including other linkers and related structures, such as the linkers shown in **Formulas 1, 2** and **3** can also be made substantially in accordance with **Reaction Scheme 3**, resulting in modified solid support compositions including the quencher moiety, such the ones shown in **Formula 4** and **Formula 5**.

The modified solid support compositions including the quencher moiety of structure **12** and of **Formula 4** and **5** are used for preparing 3'-quencher conjugates, allowing the introduction of a fluorophore at the 5'-end with the appropriate phosphoramidite, or post-synthetically with a fluorophore containing a reactive group. In **Reaction Scheme 3** and in **Formula 4** and **Formula 5** the symbols are defined as above. It should be understood that other solid supports (such as polystyrene) and other cleavable linker systems (in addition to the succinate linker shown) can also be prepared in accordance with these generic teachings and therefore are also within the scope of the invention.



Formula 4



Formula 5

Minor Groove Binder Quencher Reagents for Oligonucleotide Synthesis

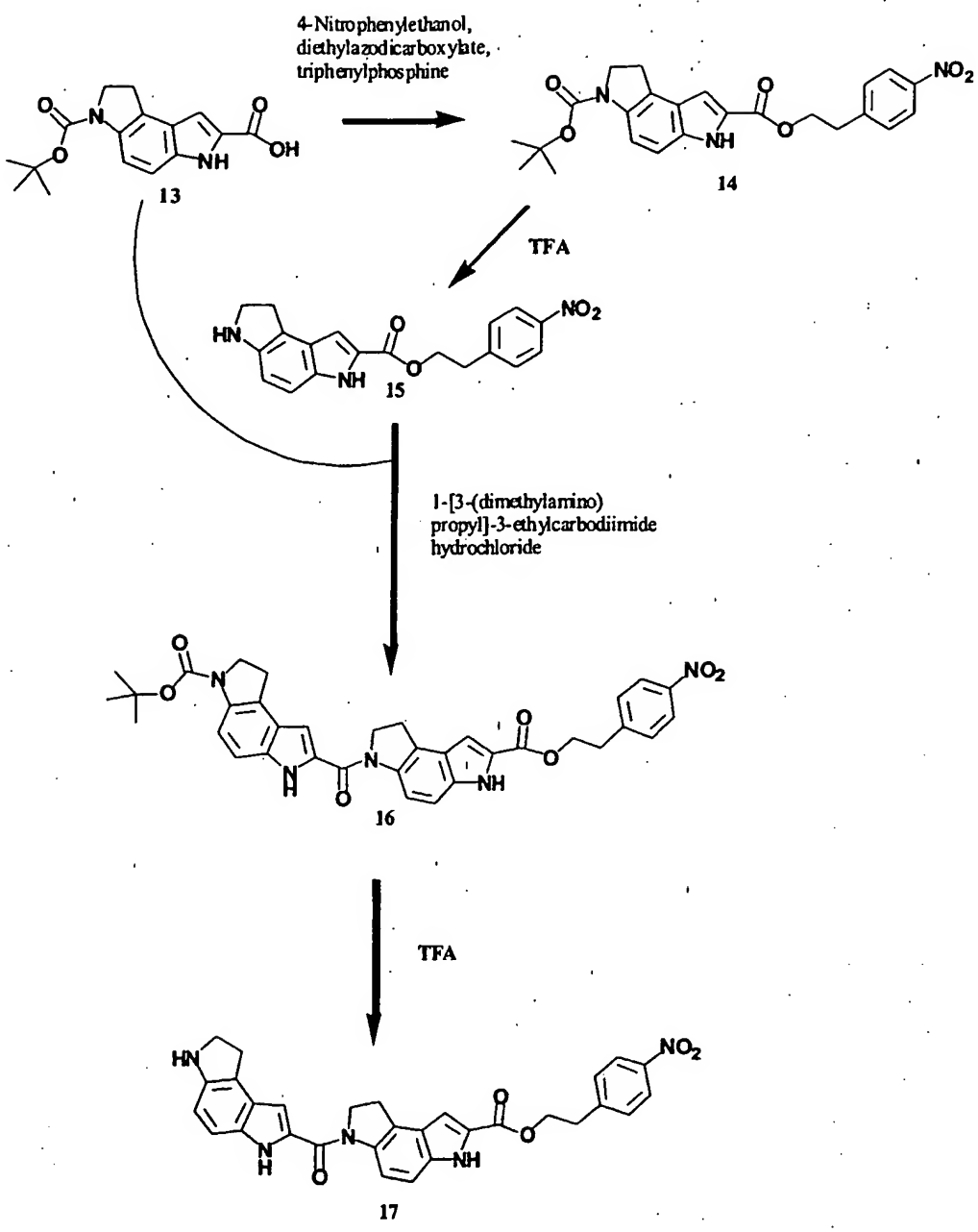
In one embodiment a minor groove binder (MGB) is attached to controlled pore glass (CPG) through a cleavable linker. A quencher moiety,

based on the 4-(phenyldiazenyl)phenylamine structure, is attached through a linker molecule to the MGB. The linker molecule also contains a hydroxyl group blocked with DMTr (or like) blocking group. After removal of the DMTr group, an oligonucleotide is synthesized on an automated oligonucleotide synthesizer by step-wise attachment of nucleotide units to the hydroxyl group. A fluorophore is introduced at the 5'-end with the appropriate phosphoramidite, or post-synthetically with a fluorophore containing a reactive group, to yield an ODN having an attached fluorescent moiety (FL), quencher (Q) and MGB (FL-ODN-Q-MGB). In this connection it is noted that the synthesis of MGBs and their attachment to ODNs is well known (see for example United States Patent No. 5,801,155, 09/539,097 and 09/141,764; all of which are expressly incorporated herein by reference.

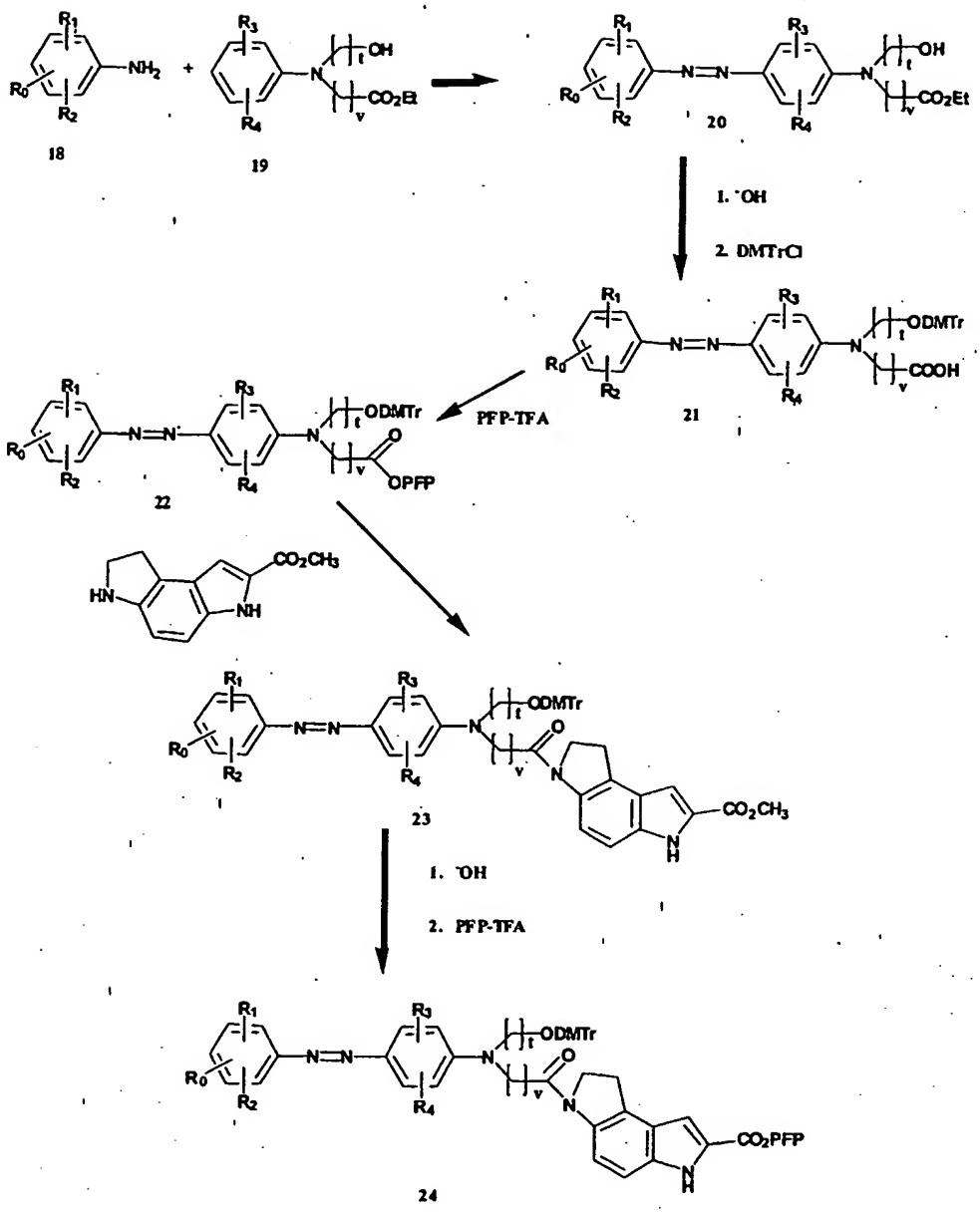
In a preferred embodiment the MGB is 3-{[3-(pyrrolo[4,5-e]indolin-7-ylcarbonyl)pyrrolo[4,5-e]indolin-7-yl]carbonyl}pyrrolo[3,2-e]indoline-7-carboxylic acid (DPI_3). The synthesis of the covalently bound "aggregate" FL-ODN-Q-DPI_3 requires five phases, described below. The first phase, shown in **Reaction Scheme 4**, is the synthesis of an intermediate, 2-(4-nitrophenyl)ethyl 3-(pyrrolo[4,5-e]indoline-7-carbonyl)pyrrolo[4,5-e]indoline-7-carboxylate (DPI_2-NPC) **17**. The second phase, shown in **Reaction Scheme 5**, is the synthesis of Q-DMTr-DPI-CO_2PFP **24** where a quencher is coupled through a linker to a pyrrolo[3,2-e]indoline-7-carboxylic acid unit (DPI). Here, and in the reaction schemes PFP stands for the pentafluorophenyl or pentafluorophenyloxy group, as the context requires. In the third phase, shown in **Reaction Scheme 6**, DMTr-Q-DPI_3-PFP **25a** is synthesized from **17** and **24**. In the fourth phase **25a** is coupled to CPG to yield a DMTr-Q- DPI_3-CPG **29**, and in the fifth phase **29** is used on an automated oligonucleotide synthesizer to stepwise attach nucleotide units and to provide, after removal from the CPG, the product FL-5'-ODN-3'-Q-DPI_3 **30**.

The fourth and fifth phases of these synthetic process are shown in

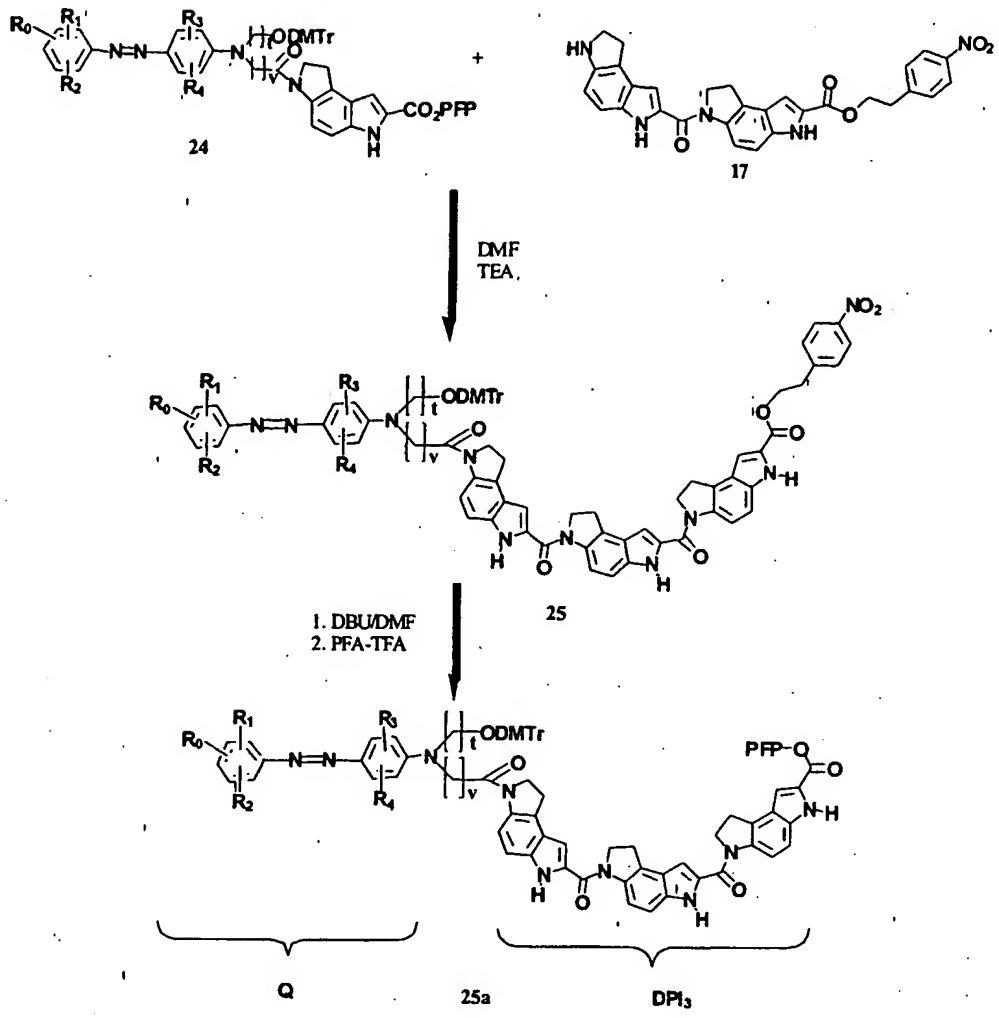
Reaction Scheme 7. Experimental conditions for this sequence (phases 1 through 5) are described below.



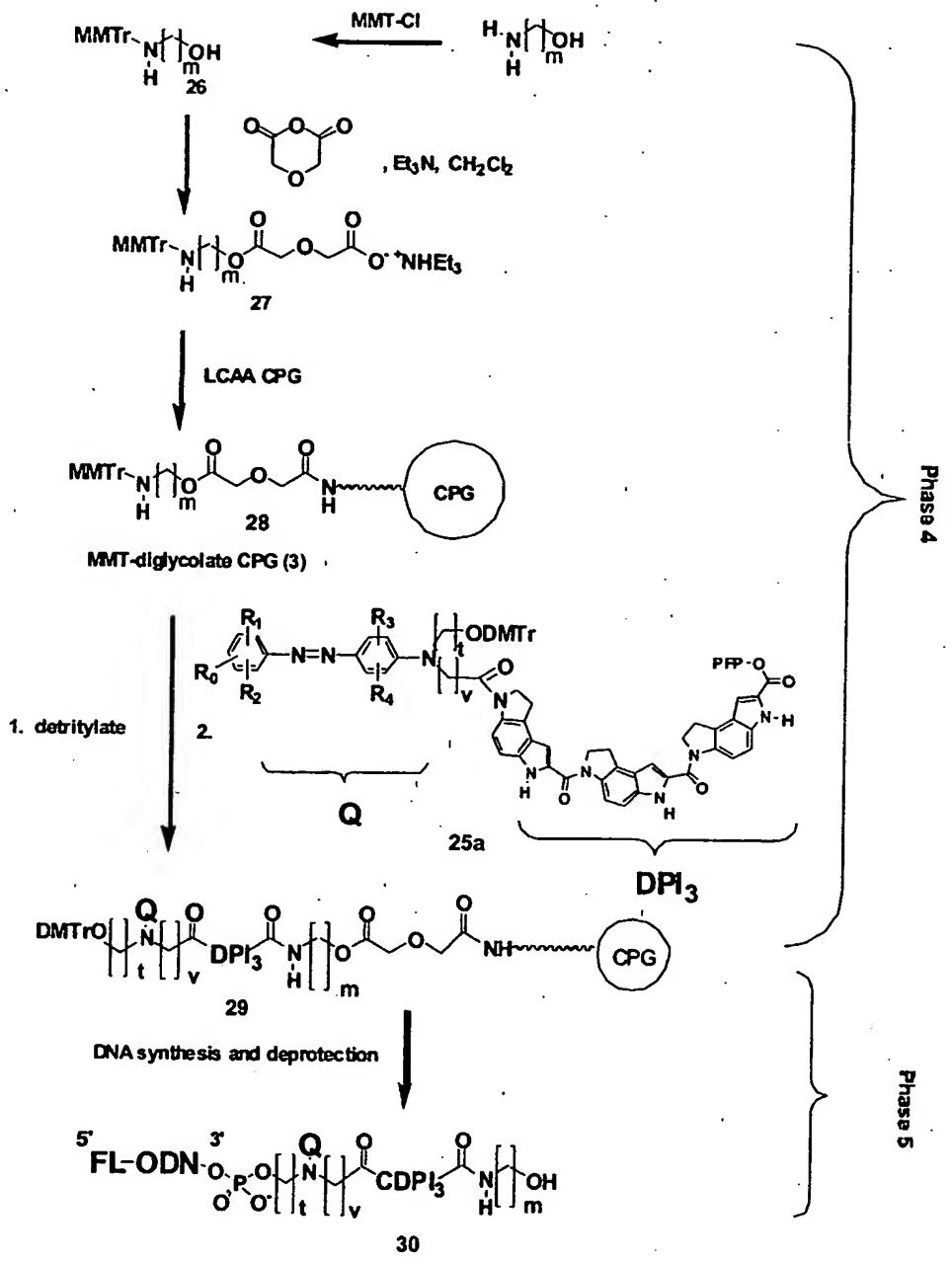
REACTION SCHEME 4



REACTION SCHEME 5



REACTION SCHEME 6



REACTION SCHEME 7

Describing these phases or reactions now in more detail, Q-DPI_3 moiety **25** (phase 3) is synthesized by the reaction of two intermediates, **17** and **24** as shown in **Reaction Scheme 6**. The first intermediate DPI_2-NPE **17** is made as shown in **Scheme 4**. DPI-tBoc **13** was reacted with p-nitrophenylethanol in the presence of diethylazodicarboxylate (DEAD) and triphenylphosphine to yield the di-ester **14**. Compound **14** was first treated with trifluoroacetic acid (TFA) to yield **15**, and then conjugated with **13** in

the presence of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride to give the 4-nitrophenyl ester of DPI_2 **16** in good yield. Reaction of **16** with TFA gives the p-nitrophenethyl ester of DPI_2 **17**. The second intermediate DPI-Q **24** (phase 2) is synthesized as shown in **Reaction Scheme 5**. A substituted nitroaniline **18** (available commercially or in accordance with the chemical literature) is diazotized in the presence of nitrous acid and is coupled to a substituted aniline **19** (available commercially or in accordance with the chemical literature) to form the azo intermediate quencher molecule **20**. Alkaline hydrolysis of the ethyl ester **20** followed by the treatment with DMTrCl gives the DMTr-Q **21**, that is subsequently activated with pentafluorophenyl trifluoroacetate to yield **22**. Reaction of **22** with DPI-methyl ester gives the Q-DMTr-DPI methylester **23**. Compound **23** is then treated with alkali to hydrolyze the methyl ester and then activated with PFP-TFA to yield Q-DMTr-DPI PFPester **24**. In **Reaction Scheme 5** the symbols R_o, R_1 through R_4, v and t are defined as above.

Referring now to **Reaction Scheme 6**, where the symbols are also defined as above, DMTr-Q-DPI_3-PFP **25a** (third phase) is synthesized first by reacting the activated quencher **24** (DMTr-Q-DPI PFP) with DPI_2-NPC **17** to yield the p-nitrophenylethyl ester **25**, which is converted to the active ester **25a**, first by treatment with base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to remove the p-nitrophenylethyl moiety and then treatment with 2,3,4,5,6-pentafluorophenyl trifluoroacetate (PFP-TFA).

The synthesis of DMTr-Q-DPI_3-CPG **29** (phase four) is shown in **Reaction Scheme 7.** In this synthetic sequence the improved quencher molecule becomes attached through a cleavable diglycolate linker to controlled pore glass beads (CPG). Specifically, aminopropanol, or a homolog thereof, is reacted successively with monomethoxytrityl chloride (MMTr-Cl) and then with diglycolic anhydride to form MMT-blocked aminopropanol **26** (or homolog) and MMT-diglycolate **27**, respectively. The symbol **m** is defined as an integer having the values 2 to 20. For the presently

preferred aminopropanol **m** is 3. The remaining symbols in this scheme are defined as above. Reaction of **27** with long chain aminoalkyl CPG in the presence of activating agents (HOBT and HBTU), yields the MMT-diglycolate-CPG **28**, that is converted after detritylation and reaction with **25a** to DMTrO-Q-DPI_3-CPG **29.**

In phase 5, still shown in **Reaction Scheme 7** oligonucleotide synthesis is performed with the aid of an automated DNA synthesizer, and a fluorophore is attached at the 5'-end of the ODN, using either a fluorphore-phosphoramidite or a fluorophore containing a reactive group, to yield the FL-ODN-Q-DPI_3 **30** conjugate.

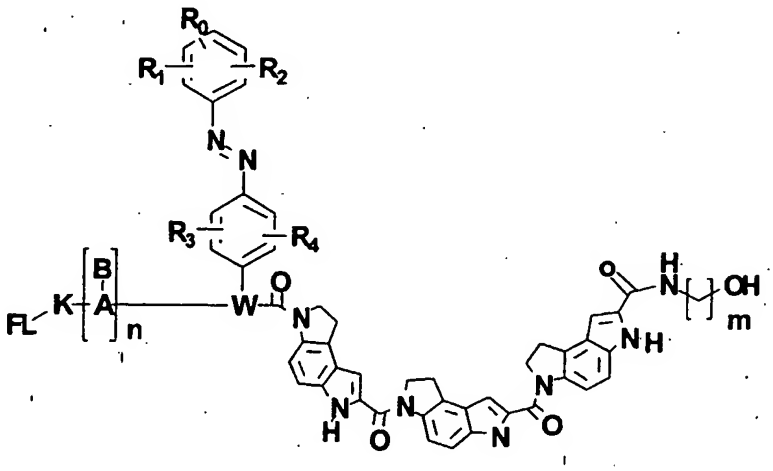
The FL-ODN-Q-DPI_3 **30** conjugate can also be synthesized by an alternative synthetic route which is not specifically illustrated in the reaction schemes. In this alternative route DPI_3-methyl ester (obtained in accordance with *Boger et al.*, J. Org. Chem., 52: 1521- (1987) incorporated herein by reference) is first reacted with compound **22** and then with alkali to give Q-DPI_3-methyl ester and Q-DPI_3-COOH, respectively. The latter compound is then activated with pentafluorophenyl trifluoroacetate, to yield **25a,** which is then used in the reactions shown in **Scheme 7,** to yield **30.**

FL-ODN-Q and FL-ODN-Q-MGB Probes

A general structure of a preferred embodiment of Fl-ODN-Q-DPI_3 conjugates is shown in **Formula 6** where:

FL is a fluorophore with emission wavelengths in the range of about 300 to about 800 nm and more preferably 400 to 700 nm; K is a linker containing between 1 and 30 atoms, which include any of C, O, N, S, P and H; $\mathbf{[A\text{-}B]_n}$ symbolizes a DNA, RNA or PNA or any combination thereof, where **A** is the sugar phosphate backbone (including modified sugars and modified phosphates), **B** is the heterocyclic base, and **n** is the number of nucleotide units. **B** can independently be any of the purine- and pyrimidine-; pyrazolo[3,4-d]pyrimidine-, 7-substituted pyrazolo[3,4-d]pyrimidine- , 7-deazapurines, 7-substiuted 7-deazapurines, modified purine- and pyrimidine-

bases, and the oligonucleotide or nucleic acid can include any combinations of these bases. W is a linker of the length of 0 to approximately 30 atoms, selected from the group consisting of C, O, N, S, P and H, furthermore W is a substituted branched aliphatic chain, or a substituted ring structure or a combined substituted aliphatic and ring structure; R_0, R_1, R_2, R_3 and R_4 are as described previously and m=1 to 20.



Formula 6

Syntheses of PNA and PNA/DNA chimeras are known in the art and can generally speaking be performed in accordance with the publications *Uhlmann et al.*, Angew. Chem. Inter. Ed., 37:2796-2823 (1998); *Mayfield et al.*, Anal. Biochem., 401-404 (1998) which are incorporated herein by reference.

A still more preferred embodiment within the scope of **Formula 6** is one where W is $-(CH_2)_3N(-)-(CH_2)_3-$; $R_0=NO_2$; R_1=-Cl; $R_2=R_3=R_4=H$; K is a 6 carbon linker and m=3.

Conjugate probes of the present invention containing a fluorescent reporter-quencher pair are, generally speaking, used in conjunction with the amplification of target polynucleotides, frequently in methods utilizing PCR, as described for example by *Holland et al.* Proc. Acad. Sci., 88: 7276-

7280(1991) and *Wittwer et al.*, Biotechniques, 22: 176-181 (1997) which are incorporated herein by reference. The binding site of the conjugate probe is located between the PCR primers used to amplify the target polynucleotide.

Use of the conjugate oligonucleotide probes according to the present invention for detection of target oligonucleotide sequences provides several advantages over prior-art reporter quencher groups and combinations. For example, the quenchers including the 4-[4-nitrophenyl)diazinyl]phenylamine structure in accordance with the present invention gave larger signal to noise ratios (S/N) in probes with either FAM or TAMRA serving as reporters than dabcyl as a quencher. Furthermore, the quenchers in accordance with the invention show a broader absorbance range than dabcyl, allowing efficient quenching of a broad range of fluorophores. In addition, in MGB-oligonucleotide conjugates have improved hybridization characteristics, an improved quencher showed about 30-fold increase in S/N ratio with TAMRA compared to a standard probe (no DPI_3) with dabcyl. Reagents of the present invention allow the introduction of the quencher during automated oligonucleotide synthesis. (Dabcyl phosphoramidite is commercially available; (Glen Research, Sterling, VA))

It should be understood that, generally speaking, for the purpose of this invention, an oligonucleotide comprises a plurality of nucleotide units, a 3' end and a 5'end. The oligonucleotide may contain one or more modified bases other than the normal purine and pyrimidine bases, as well as modified internucleotide linkages capable of specifically binding target polynucleotide through Watson-Crick base pairing, or the like. In addition, oligonucleotides may include peptide oligonucleotides (PNAs) or PNA/DNA chimeras, the synthesis of which is known and can be performed for example in accordance with the publications *Uhlmann et al.*, Angew. Chem. Inter. Ed., 37:2796-2823 (1998) and *Mayfield et al.*, Anal. Biochem., 401-404 (1998); all of which are expressly incorporated herein by reference.

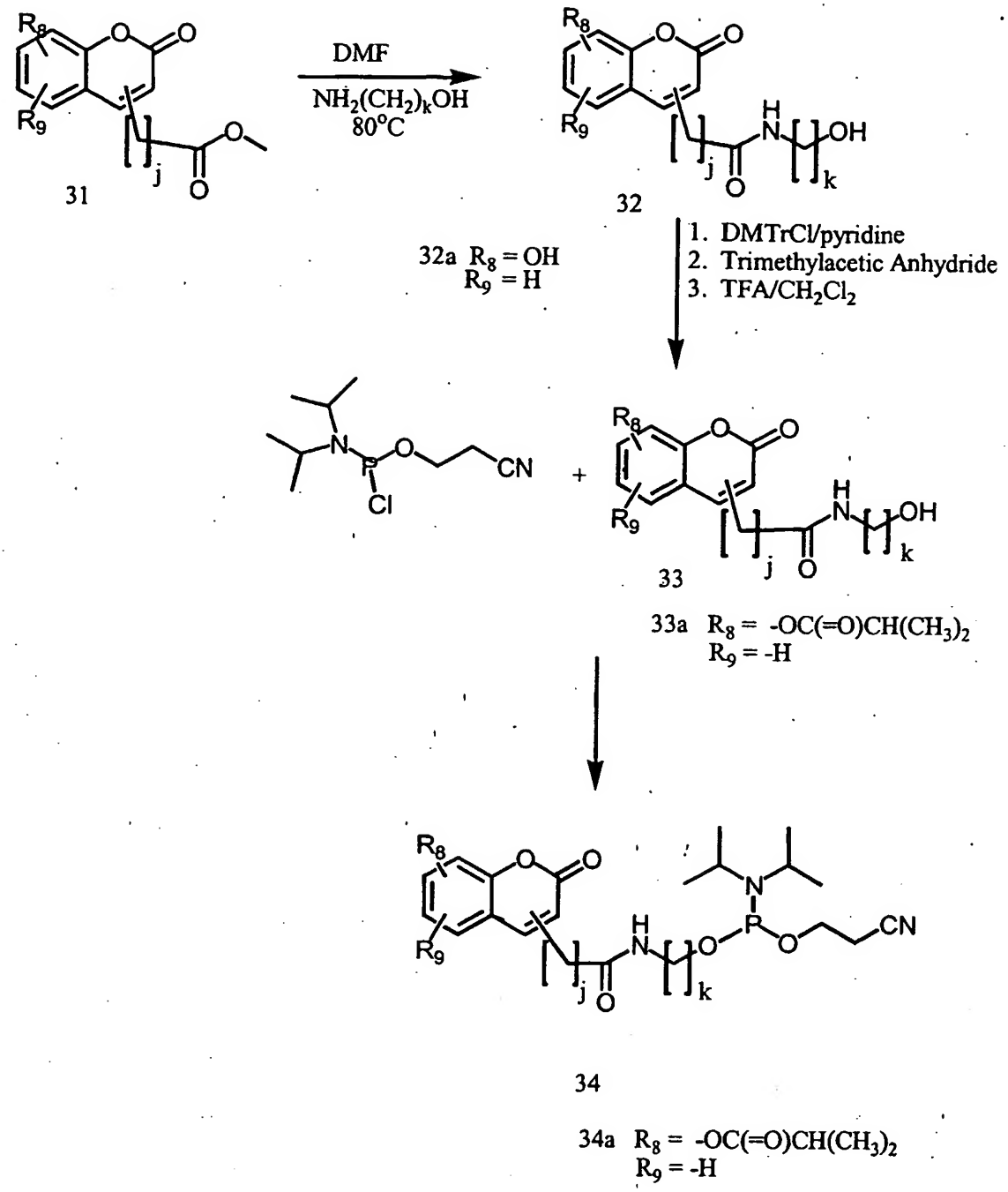
Generally, the oligonucleotide probes of the invention will have a

sufficient number of phophodiester linkages adjacent to the 5' end to allow 5'-3' exonuclease activity to allow efficient cleavage between the quencher and fluorophore molecules in the probe. An adequate number in this regards is approximately between 1 and 100.

FLUOROPHORE REAGENTS

Coumarin Phosphoramidite Reagents

Fluorescent dyes which have emission wavelengths shorter than the green fluorescent dye FAM also have utility in DNA probe based assays. However in the prior art these probes have been less popular since excitation with laser light sources is less feasible than with longer wavelengths. To the best knowledge of the present inventors, to date, there have been no reports of DNA synthesis reagents that contain blue fluorescent dyes. An example of such a phosphoramidite reagent containing a preferred coumarin fluorophore and which is suitable for DNA synthesis, is shown in **Reaction Scheme 8**, as compound **34**. In the phosphoramidite reagent **34**, R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, a blocking group compatible with oligomer synthesis and which can be removed under acid or alkaline conditions; or a group that contains between 1 and 10 carbon atoms, j and k independently are 1 to 10. As can be seen the reagent **34** includes a covalently linked coumarin chromophore which emits light at 458 nm. DNA probes containing this coumarin chromophore were prepared and gave the desired fluorescent emission properties.



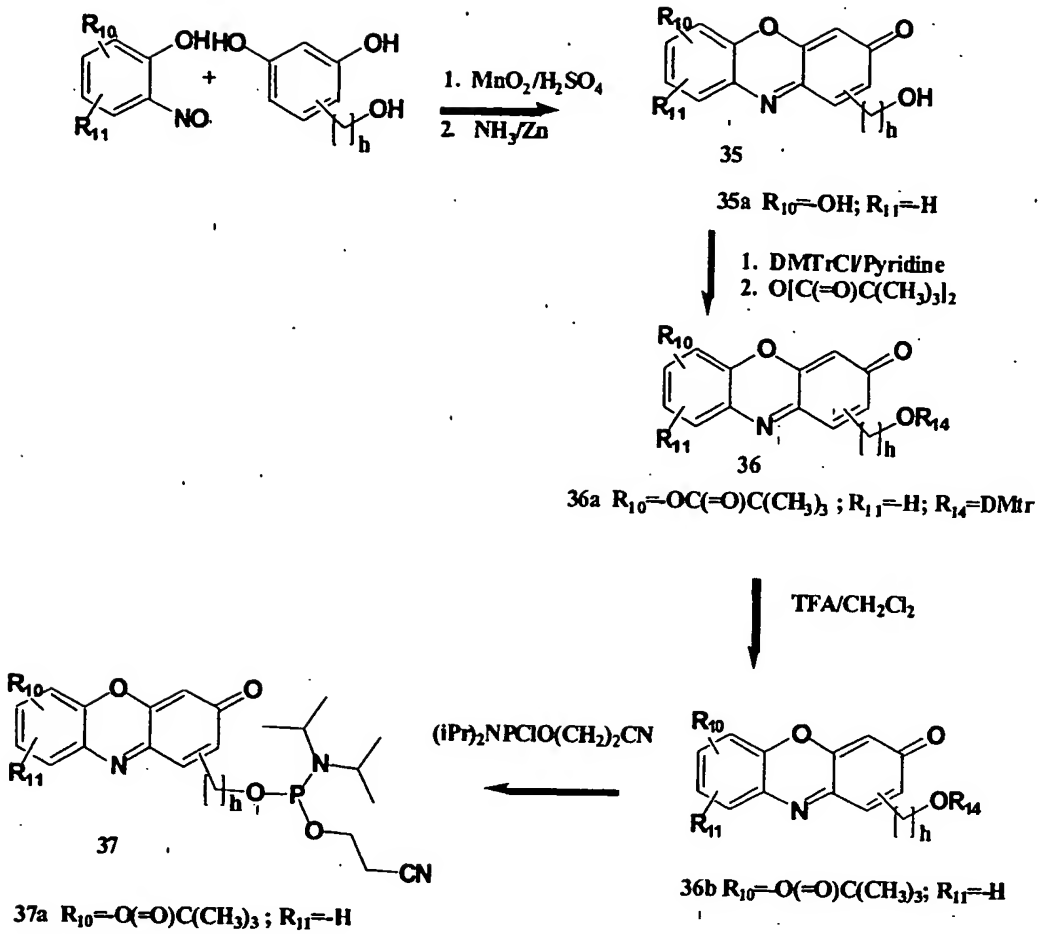
REACTION SCHEME 8

Referring now to **Scheme 8** in general terms and also in an example that provides the specific phosphoramidite reagent **34a**, a hydroxyl substituted (2-oxo-2H-chromen-4-yl)-alkylcarboxyl methyl ester (**31**) is obtained according to the publication *Baker et al.* (J.Chem.Soc.; 170, 173 (1950)) incorporated herein by reference. Compound **31** is converted to the alkanol

derivative **32** (specifically to **32a** where R_8 is -OH and R_9 is -H) by reaction with an aminoalkanol at 80°C. Reaction of **32** first with DMTrCl and then with trimethylacetic anhydride followed by the removal of the DMTr blocking group gives a pivaloate derivative **33,** in the specific example **33a** where $\mathbf{R_8}$ is $-OC(=O)CH(CH_3)_2$ and R_9 is -H. Reaction of **33** with 2-cyanoethyl diisopropylchlorophosphoramidite gives reagent **34** (specifically **34a** where $\mathbf{R_8}$ is $-OC(=O)CH(CH_3)_2$, R_9 is -H). The reagent **34** is used for incorporating the coumarin fluorophore into the 5'-terminus of DNA probes. It is noteworthy that removal of the protecting groups during automated oligonucleotides synthesis proceeded well, resulting in high yields. The symbols **j** and **k** in **Scheme 8** are defined as 0 to 20 and 1 to 20, respectively.

Resorufin Phosphoramidite

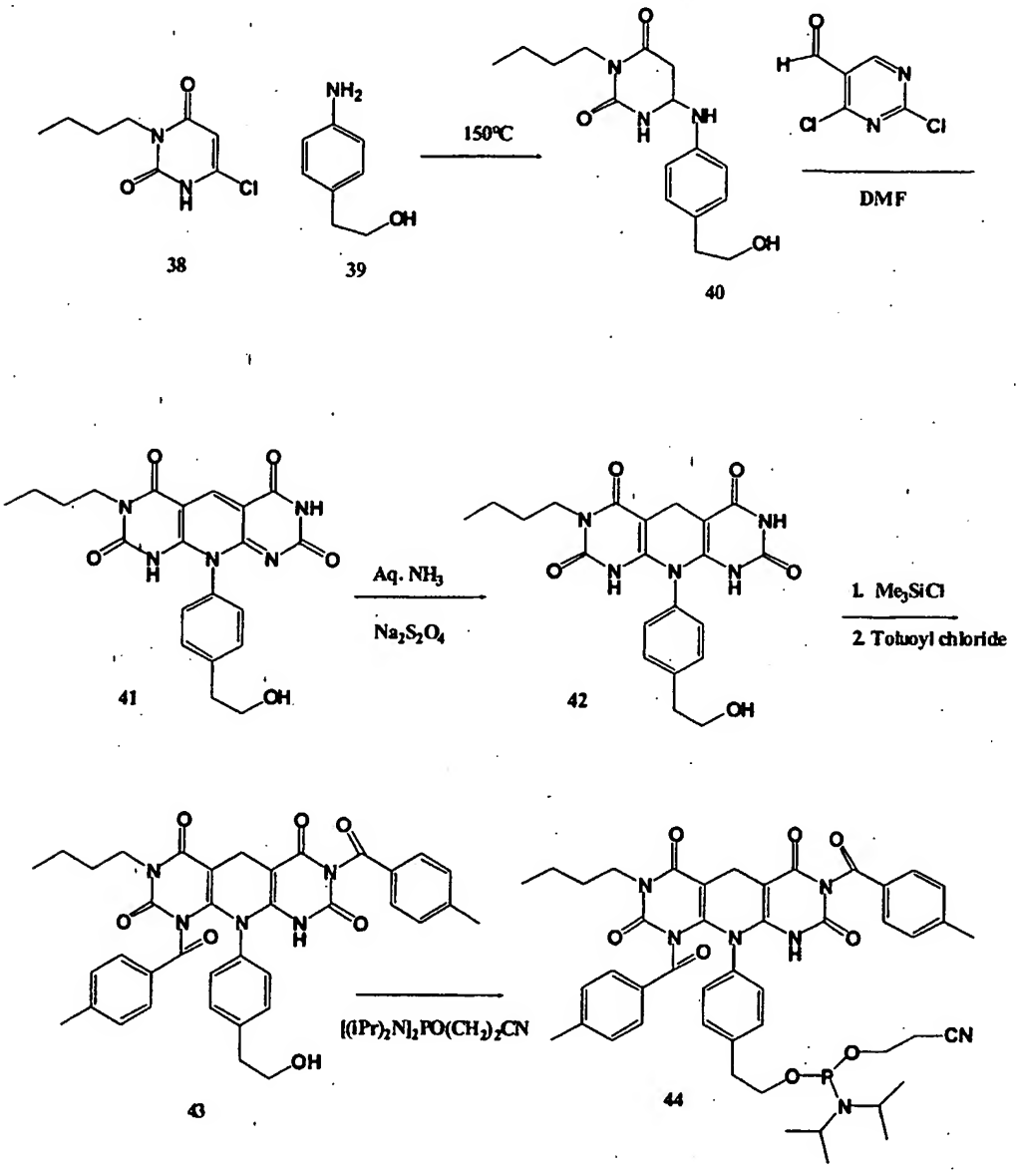
Another new class of DNA synthesis reagents are based on the 7-hydroxy-3H-phenoxazin-3-one chromophore present in the parent compound (resorufin) and have emission wavelength (595 nm) that is easily distinguished from FAM emission. In accordance with the invention the chromophore is synthesized in such a way as to incorporate a linker structure for further functionalization to the desired phosphoramidite reagents. The preparation of preferred examples of these reagents **37** suitable for DNA synthesis, is shown in **Reaction Scheme 9**. Generally speaking in reagent **37** R_{10} and R_{11} independently are H, $-OR_{12}$, $-NHR_{13}$, halogen,$-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$, O-alkyl or O-alkanoyl where the alkyl or alkanoyl group has 1 to 10 carbons, or –CN where n= 0 to 5; h=1 to 20; and $\mathbf{R_{12}}$ and $\mathbf{R_{13}}$ are blocking groups compatible with ODN synthesis. $\mathbf{R_{14}}$ in the scheme is H or DMTr.



REACTION SCHEME 9

As is shown in the example of **Scheme 9** in general terms and also for a specific example, reaction of nitrosorecorcinol derivative (commercially available or synthesized in accordance with the state-of-the-art) and of 4-(3-hydroxypropyl)benzene-1,3-diol (obtained in accordance with Forchiassin *et al. J. .Heterocyc. Chem.* **20**, 1983, 493-494.) and MnO_2 yielded a resazurin derivative contaminated with some resorufin derivative. This mixture was treated with NH_4OH and Zn dust to yield resorufin derivative **35** (specifically **35a** where R_{10} is OH, and R_{11} is H) contaminated with 2,3,4-trihydro-2H-pyrano[3,2-b]phenoxazin-9-one as major impurity. The latter mixture was treated with DMTrCl and pyridine, and then with trimethylacetic acid anhydride. The product **36** was then subjected to purification by chromatography on silica gel to give the DMTr-protected derivative of **36a**

(where R_{10} is -OC(=O)C(CH $_3)_3$, R_{11} is H and R_{14} is DMTr). The pure DMTr-derivative was treated with TFA/CH_2Cl_2 to yield a single product **36b** after silica gel chromatography. Treatment of **36** (where R_{10} is -OC(=O)C$(CH_3)_3$, R_{11} and R_{14} are H) with 2-cyanoethyl diisopropylchlorophosphoramidite gave the desired phosphoramidite reagent **37** (specifically **37a**) that is utilized to introduce the fluorophore into a desired ODN.



REACTION SCHEME 10

PPT Phosphoramidite

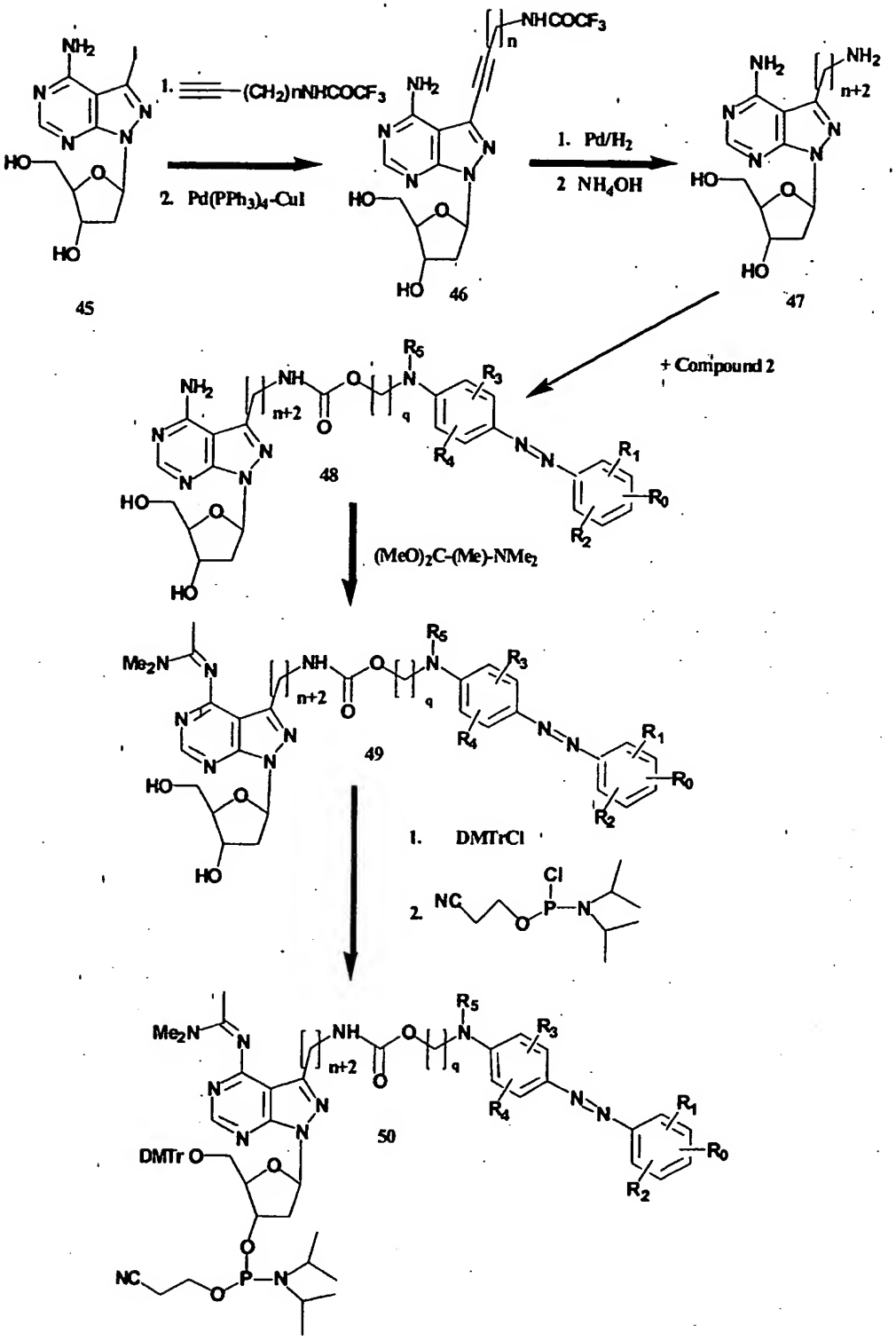
The synthesis of a phosphoramidite reagent incorporating a purple fluorescent dye PPT **44** having excitation and emmision wavelengths of 384 and 400 nm, respectively is shown in **Reaction Scheme 10** and in **Example X.** In accordance with this scheme 6-chloro-3-n-butyluracil **38** and 2-(4-aminophenyl)ethanol **39** are reacted to yield the phenyl substituted uracil derivative **40**. The compounds **38** and **39** can be obtained in accordance with the state-of-the-art and the chemical literature. Reaction of **40** with 5-formyl-4,6-dichloro pyrimidine in DMF at room temperature affords the tricyclic heterocycle **41**. Reduction of **41** in $NH_3/Na_2S_2O_4$ yields **42** which is thereafter blocked as the toluoyl-derivative **43.** In the final step **43** is reacted with 2-cyanoethyl diisopropylchlorophosphoramidite to yield the reagent PPT cyanoethyl phoporamidite **44** that is used to introduce the PPT fluorophore into an ODN.

In still further embodiments, the fluorophores coumarin, resorufin and PPT substituted with an alkylcarboxyl group serve as starting materials for the synthesis of the corresponding phosphoramidite reagents. The fluorophores coumarin, resorufin and PPT substituted with an alkylcarboxyl group are either commercially available or can be synthesized in accordance with the state-of-the-art. These compounds are activated on the alkylcarboxyl group as the pentafluorophenyl esters. The activated esters are used to attach these dyes to amine modified oligonucleotides.

Similarly, in still other embodiments, dUTP-labeled quenchers or fluorophores are obtained for example in accordance with the teachings of United States Patent No. 5,328,824). Furthermore, the phosphoramidite of 7-labeled pyrazolo[3,4-d]pyrimide-labeled quenchers or fluorophores are synthesized according to the teaching of 5,824,796 (incorporated herein by reference) and can be used for labeling of oligonucleotides.

PPG Red Dye-based and Other Phosphoramidite Reagents for Oligonucleotide Synthesis.

In another embodiment the red dye 13 quencher is attached to the 3-position of pyrazolo[5,4-d]pyrimidines (PP) or the 5-position of a pyrimidine. Referring now to **Scheme 11** itself, the starting material is 5-(4-amino-3-iodopyrazolo[5,4-d]pyrimidinyl)-2-(hydroxymethyl)oxolan-3-ol **45** which is available in accordance with the publication *Seela et al.* J. Chem. Soc., Perkin. Trans., 1 (1999, 479-488) incorporated herein by reference. Compound **45** is first reacted with N-propynyl-2,2,2-trifluoroacetate (or a homolog thereof where in the scheme **n** is 1 to 10) and then with $Pd(PPh_3)_4$-CuI to give the alkyne derivative **46**. Pd/H_2 reduction of **46** followed by ammonium hydroxide treatment gives the aminoalkyl derivative **47** (PPA'). Reaction of PPA' with compound **2** (available as disclosed in connection with **Reaction Scheme 1)** yielded substituted PPA'-Red 13 **48**. Reaction of **48** with (1,1-dimethoxyethyl)dimethylamine blocks the amino group of the pyrazolo[5,4-d]pyrimidine to yield **49**. **49** is first reacted with DMTrCl and then with 2-cyanoethyl diisopropylchlorophosphoramidite to give the DMTrCl blocked PPA'-Red 13 phosphoramidite **50**.



REACTION SCHEME 11

In still other embodiments starting with the deoxyriboside of 6-amino-5-hydroxy-3-iodo-pyrazolo[5,4-d]pyrimidin-4-one (3-Iodo-PPG) the phosphoramidite reagent containing the Red 13 dye covalently linked to the 3-Iodo-PPG moiety is synthesized with reactions analogous to those shown in **Reaction Scheme 11**. Similarly starting with 5-aminopropyldeoxyuridine the phosphoramidite reagent containing the Red 13 dye covalently linked to 5-aminopropyl-deoxyuridine is synthesized.

It will be clear to those skilled in the art in light of the foregoing disclosure that the pyrazolopyrimidine-Red-13- or uridine-Red 13-based phosphoramidites within the scope of this invention may contain various linkers between the pyrazolopyrimidine and uracil bases and the Red 13 quenchers, to the full extent such linkers are available in accordance with the state of the art and this disclosure.

EXAMPLES

The methods and compositions of the present invention can be used with a variety of techniques, both currently in use and to be developed, in which hybridization of an oligonucleotide to another nucleic acid is involved. These include, but are not limited to, techniques in which hybridization of an oligonucleotide to a target nucleic acid is the endpoint; techniques in which hybridization of one or more oligonucleotides to a target nucleic acid precedes one or more polymerase-mediated elongation steps which use the oligonucleotide as a primer and the target nucleic acid as a template; techniques in which hybridization of an oligonucleotide to a target nucleic acid is used to block extension of another primer; techniques in which hybridization of an oligonucleotide to a target nucleic acid is followed by hydrolysis of the oligonucleotide to release an attached label; and techniques in which two or more oligonucleotides are hybridized to a target nucleic acid and interactions between the multiple oligonucleotides are measured. The

conditions for hybridization of oligonucleotides, and the factors which influence the degree and specificity of hybridization, such as temperature, ionic strength and solvent composition, are well-known to those of skill in the art. *See*, for example, *Sambrook et al., supra*; *Ausubel et al., supra*; *Innis et al.* (eds.) PCR Protocols, Academic Press, San Diego, 1990; *Hames et al.* (eds.) Nucleic Acid Hybridisation: A Practical Approach, IRL Press, Oxford, 1985; and *van Ness et al.* (1991) *Nucleic Acids Res.* **19**:5143-5151.

Hybridization Probes

In one application of the present invention, one or more FL-oligonucleotide conjugates are used as probe(s) to identify a target nucleic acid by assaying hybridization between the probe(s) and the target nucleic acid. A probe may be labeled with any detectable label of the present invention, or it may have the capacity to become labeled either before or after hybridization, such as by containing a reactive group capable of association with a label or by being capable of hybridizing to a secondary labeled probe, either before or after hybridization to the target. As a basis of this technique it is noted that conditions for hybridization of nucleic acid probes are well-known to those of skill in the art. *See*, for example, *Sambrook et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory Press (1989); *Ausubel et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons (1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996); *Hames et al.* (eds.) Nucleic Acid Hybridization: A Practical Approach, IRL Press, Oxford, 1985; and *van Ness et al. Nucleic Acids Res.* **19**:5143-5151(1991).

Hybridization can be assayed (*i.e.*, hybridized nucleic acids can be identified) by distinguishing hybridized probe from free probe by one of several methods that are well-known to those of skill in the art. These include, but are not limited to, attachment of target nucleic acid to a solid support, either directly or indirectly (by hybridization to a second, support-bound probe or interaction between surface-bound and probe-conjugated

ligands) followed by direct or indirect hybridization with probe, and washing to remove unhybridized probe; determination of nuclease resistance; buoyant density determination; affinity methods specific for nucleic acid duplexes (*e.g.*, hydroxyapatite chromatography); interactions between multiple probes hybridized to the same target nucleic acid; *and other known techniques.* See, for example, *Falkow et al.*, United States. Patent No. 4,358,535; *Urdea et al.*, United States Patent Nos. 4,868,105 and 5,124,246; *Freifelder*, *Physical Biochemistry*, Second Edition, Freeman & Co., San Francisco, 1982; Sambrook, et al., supra; Ausubel et al., supra; and Hames et al., supra.

Assays Utilizing Labeled Probes, Hydrolyzable Probe and Labeled Primers

Additional applications for oligonucleotide conjugates containing a fluorophore and quencher are found in assays in which a labeled probe is hybridized to a target and/or an extension product of a target, and a change in the physical state of the label is effected as a consequence of hybridization. A probe is a nucleic acid molecule that is capable of hybridizing to a target sequence in a second nucleic acid molecule. By way of example, one assay of this type, the hydrolyzable probe assay, takes advantage of the fact that many polymerizing enzymes, such as DNA polymerases, possess intrinsic 5'-3' exonucleolytic activities. Accordingly, if a probe is hybridized to a sequence that can serve as a template for polymerization (for instance, if a probe is hybridized to a region of DNA located between two amplification primers, during the course of an amplification reaction), a polymerizing enzyme that has initiated polymerization at an upstream amplification primer is capable of exonucleolytically digesting the probe. Any label attached to such a probe will be released, if the probe is hybridized to its target and if amplification is occurring across the region to which the probe is hybridized. Released label is separated from labeled probe and detected by methods well-known to those of skill in the art, depending on the nature of the label. For example, radioactively labeled fragments can be separated by thin-layer chromatography and detected by autoradiography; while fluorescently-labeled fragments can be detected by irradiation at the appropriate excitation wavelengths with observation at the

appropriate emission wavelengths. This basic technique is described for example in United States Patent No. 5,210,015 incorporated herein by reference.

In a variation of this technique, a probe contains both a fluorescent label and a quenching agent, which quenches the fluorescence emission of the fluorescent label. In this case, the fluorescent label is not detectable until its spatial relationship to the quenching agent has been altered, for example by exonucleolytic release of the fluorescent label from the probe. Thus, prior to hybridization to its target sequence, the dual fluorophore/quencher labeled probe does not emit fluorescence. Subsequent to hybridization of the fluorophore/quencher-labeled probe to its target, it becomes a substrate for the exonucleolytic activity of a polymerizing enzyme which has initiated polymerization at an upstream primer. Exonucleolytic degradation of the probe releases the fluorescent label from the probe, and hence from the vicinity of the quenching agent, allowing detection of a fluorescent signal upon irradiation at the appropriate excitation wavelengths. This method has the advantage that released label does not have to be separated from intact probe. Multiplex approaches utilize multiple probes, each of which is complementary to a different target sequence and carries a distinguishable label, allowing the assay of several target sequences simultaneously.

The use of FL-ODN-Q-DPI_3 conjugates in this and related methods allows greater speed, sensitivity and discriminatory power to be applied to these assays. In particular, the enhanced ability of MGB-oligonucleotide conjugates to allow discrimination between a perfect hybrid and a hybrid containing a single-base mismatch facilitates the use of hydrolyzable probe assays in the identification of single-nucleotide polymorphisms and the like, as described in the publication WO 995162A2, incorporated herein by reference. Examples 13 and 14 illustrate the utility of FL-ODN-Q- DPI_3 conjugates in this type of assay. It will be clear to those of skill in the art that compositions and methods, such as those of the invention, that are capable of discriminating single-nucleotide mismatches will also be capable of discriminating between sequences that have multiple mismatches with respect

to one another.

Another application embodiment uses a self-probing primer with an integral tail, where the quencher/fluorophore is present in the hairpin, that can probe the extension product of the primer and after amplification hybridizes to the amplicon in a form that fluoresces. The probing of a target sequence can thereby be converted into a unimolecular event (Whitcombe, D. et al., Nat. Biotech., 17: 804-807 (1999)).

Fluorescence Energy Transfer

In further applications of the novel compositions of this invention, oligonucleotide conjugates containing a fluorophore/quencher pair (FL-ODN-Q) are used in various techniques which involve multiple fluorescent-labeled probes. In some of these assays changes in properties of a fluorescent label are used to monitor hybridization. For example, fluorescence resonance energy transfer (FRET) has been used as an indicator of oligonucleotide hybridization. In one embodiment of this technique, two probes are used, each containing a fluorescent label and a quencher molecule respectively. The fluorescent label is a donor, and the quencher is an acceptor, wherein the emission wavelengths of the donor overlap the absorption wavelengths of the acceptor. The sequences of the probes are selected so that they hybridize to adjacent regions of a target nucleic acid, thereby bringing the fluorescence donor and the acceptor into close proximity, if target is present. In the presence of target nucleic acid, irradiation at wavelengths corresponding to the absorption wavelengths of the fluorescence donor will result in emission from the fluorescence acceptor. These types of assays have the advantage that they are homogeneous assays, providing a positive signal without the necessity of removing unreacted probe. For further details and additional examples of these assays which are *per se* known in the art, see, for example, European Patent Publication 070685; and the publication *Cardullo et al.* (1988) *Proc. Natl. Acad. Sci. USA* **85:** 8790-8794, both of which are

incorporated herein by reference. Additional applications of the novel compositions of the present invention are in those and related techniques in which interactions between two different oligonucleotides that are both hybridized to the same target nucleic acid are, measured. The selection of appropriate fluorescence donor/fluorescence acceptor pairs will be apparent to one of skill in the art, based on the principle that, for a given pair, the emission wavelengths of the fluorescence donor will overlap the absorption wavelengths of the acceptor. The enhanced ability of DPI_3-oligonucleotide conjugates to distinguish perfect hybrids from hybrids containing a single base mismatch facilitates the use of FRET-based techniques in the identification of single-nucleotide polymorphisms and the like.

In another application of the novel compositions of the invention, the fluorescence of the FL-ODN-Q conjugate is quenched in its native state. But, after hybridization with the intended target the spatial arrangement of the fluorophore and quencher moieties are changed such that fluorescence occurs. For this basic technique see for example *Tyagi et al.*, Nat. Biotech., 16: 49-53 (1998); and United States Patent No. 5,876,930, both of which are incorporated herein by reference.

It should be understood that in addition to the fluorophores which are found in accordance with the present invention especially useful to be used with the quenchers of the invention, and which fluorophores are incorporated into ODNs in accordance with the invention, a person of ordinary skill may choose additional fluorophores to be used in combination with the quenchers of the present invention, based on the optical properties described in the literature, such as the references: *Haugland* Handbook of Fluorescent Probes and Research Chemicals, Six Edition, Eugene, OR. pp. 235-236. 1996; *Berlman*, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd, Accademic Press, New York, 1971; Du et al., PhotochemCAD. A Computer-Aided Design and Research Tool in Photochemistry, Photochem. Photobiol. 68, 141-142 (1998). Therefore the use of the novel ODN quencher

conjugates in combination with these known fluorophores is considered within the scope of the invention.

In another application, the minor groove binder, DPI_3, is coupled to a quencher in a FL-ODN-Q- $CDPI_3$ conjugate to improve signal to noise ratios (See Table 2). Preferred quenchers are the quenchers of Formula 6 and more preferred the quenchers are **8-11**, **12-16** and **30**.

Additional quenchers suitable for use in combination with the novel fluorophores (34, 37 and 44) of the invention include dabcylnitrothiazole, TAMRA, 6-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino) hexanoic acid , 6-carboxy-X-rhodamine (Rox) and QSY-7 .

Another application of the novel fluorophore/quencher pairs of the invention is to incorporate the pair into enzyme substrates, where fluorescence is quenched because of the proximity of the fluorophore and quencher. However, after an enzyme cleaves the substrate the fluorophore and quencher become separated and fluorescence is observed., An example of this technique is described below using the phosphodiesterase enzyme. It will be clear to those schooled in the art that suitable substrates containing both the novel quenchers and fluorophores can be constructed for enzymes that cleave substrates.

Oligonucleotide Arrays

In another application, FL-ODNs of the present invention are utilized in procedures employing arrays of oligonucleotides. Examples for this technique that is *per se* known in the art include sequencing by hybridization and array-based analysis of gene expression. In these procedures, an ordered array of oligonucleotides of different known sequences is used as a platform for hybridization to one or more test polynucleotides, nucleic acids or nucleic acid populations. Determination of the oligonucleotides which are hybridized and alignment of their known sequences allows reconstruction of the sequence of the test polynucleotide. For a description of these techniques see

for example, United States Patent Nos. 5,492,806; 5,525,464; 5,556,752; and PCT Publications WO 92/10588 and WO 96/17957 all of which are incorporated herein by reference. Materials for construction of arrays include, but are not limited to, nitrocellulose, glass, silicon wafers and optical fibers.

STRUCTURAL CONSIDERATIONS

The terms oligonucleotide, polynucleotide and nucleic acid are used interchangeably to refer to single- or double-stranded polymers of DNA or RNA (or both) including polymers containing modified or non-naturally-occurring nucleotides, or to any other type of polymer capable of stable base-pairing to DNA or RNA including, but not limited to, peptide nucleic acids which are disclosed by *Nielsen et al.* (1991) Science **254:**1497-1500; bicyclo DNA oligomers (*Bolli et al.* (1996) Nucleic Acids Res. **24:**4660-4667) and related structures. One or more MGB moieties and/or one or more fluorescent labels, and quenching agents can be attached at the 5' end, the 3' end or in an internal portion of the oligomer. A preferred MGB in accordance with the invention is DPI_3 and the preferred quencher is red 13 amide.

Preferred in the present invention are DNA oligonucleotides that are single-stranded and have a length of 100 nucleotides or less, more preferably 50 nucleotides or less, still more preferably 30 nucleotides or less and most preferably 20 nucleotides or less with a lower limit being approximately 5 nucleotides.

Oligonucleotide conjugates containing a fluorophore/quencher pair with or without an MGB may also comprise one or more modified bases, in addition to the naturally-occurring bases adenine, cytosine, guanine, thymine and uracil. Modified bases are considered to be those that differ from the naturally-occurring bases by addition or deletion of one or more functional groups, differences in the heterocyclic ring structure (*i.e.*, substitution of carbon for a heteroatom, or *vice versa*), and/or attachment of one or more

linker arm structures to the base. The modified nucleotides which may be included in the ODN conjugates of the invention include 7-deazapurines and their derivatives and pyrazolopyrimidines (described in PCT WO 90/14353 incorporated herein by reference); and in co-owned and co-pending application serial number 09/054,630.

Preferred base analogues of this type include the guanine analogue 6-amino-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (ppG or PPG) and the adenine analogue 4-amino-1*H*-pyrazolo[3,4-d]pyrimidine (ppA or PPA). Also of use is the xanthine analogue 1*H*-pyrazolo[5,4-d]pyrimidin-4(5*H*)-6(7*H*)-dione (ppX). These base analogues, when present in an oligonucleotide, strengthen hybridization and improve mismatch discrimination. All tautomeric forms of naturally-occurring bases, modified bases and base analogues may be included in the oligonucleotide conjugates of the invention.

Similarly, modified sugars or sugar analogues can be present in one or more of the nucleotide subunits of an oligonucleotide conjugate in accordance with the invention. Sugar modifications include, but are not limited to, attachment of substituents to the 2', 3' and/or 4' carbon atom of the sugar, different epimeric forms of the sugar, differences in the α- or β- configuration of the glycosidic bond, and other anomeric changes. Sugar moieties include, but are not limited to, pentose, deoxypentose, hexose, deoxyhexose, ribose, deoxyribose, glucose, arabinose, pentofuranose, xylose, lyxose, and cyclopentyl.

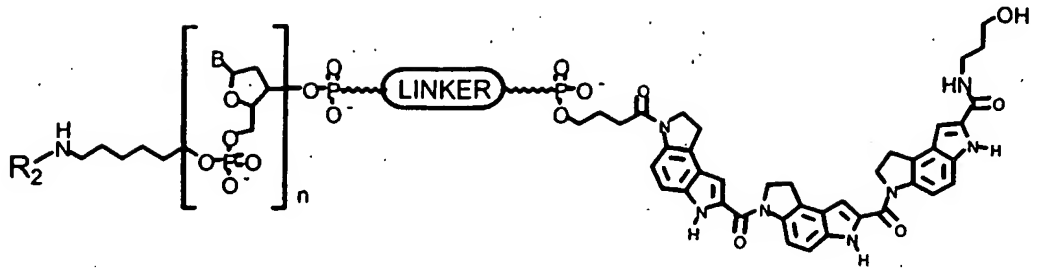
Modified internucleotide linkages can also be present in oligonucleotide conjugates of the invention. Such modified linkages include, but are not limited to, peptide, phosphate, phosphodiester, phosphotriester, alkylphosphate, alkanephosphonate, thiophosphate, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, substituted phosphoramidate and the like. Several further modifications of bases, sugars and/or internucleotide linkages, that are compatible with their use in

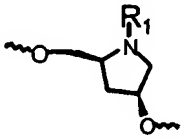
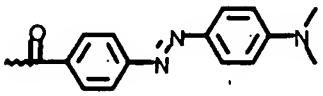
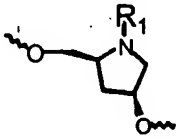
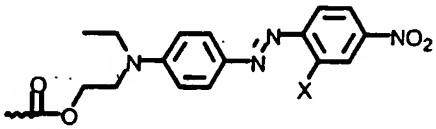
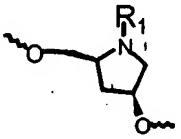
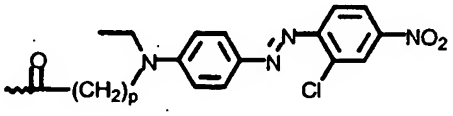
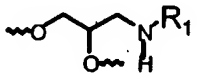
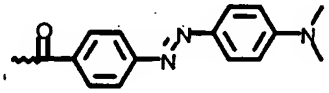
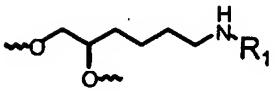
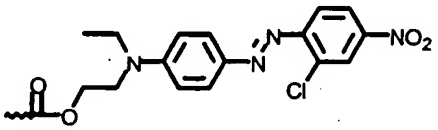
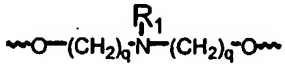
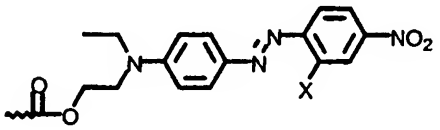
oligonucleotides serving as probes and/or primers, will be apparent to those of skill in the art.

Certain preferred embodiments of the invention involve the synthesis of numerous phophoramidites with various quencher chromophores and linkers and their incorporation at the 3'-end of fluorogenic MGB ODNs as shown in **Reaction Scheme 3**. Different fluorescent reporter groups (shown in **Reaction Scheme 7)** were also incorporated into the oligonucleotide probes and are described in the EXPERIMENTAL section. The fluorogenic properties of these ODN conjugates are described in **Table 2**. In other embodiments MGB molecules, due to their desirable improved hybridization properties, were incorporated into oligonucleotides containing both a fluorophore and a quencher, without loss in hybridization specificity, fluorescent quenching and fluoresent signal. The flat aromatic quencher residue coupled to the neighboring aromatic DPI_3 residue, have strict geometric requirements since the linker between the oligonucleotide and the DPI_3 residue must be flexible enough to allow positioning of the DPI_3 in the minor groove after DNA duplex formation.

Characteristics of Reagents of the Invention

A number of FL-ODN-Q-DPI_3 conjugates synthesized with the reagents and methods of the invention are shown in **Formulas 7** to **16**, where n specifies the number of bases in the oligonucleotide and R_2 is either FAM or TAMRA. "B" signifies a heterocyclic base attached to the deoxyribose sugar moiety.



	Linker Structure	R_1
Formula 7		
Formula 8 (X = Cl) Formula 9 (X = H)		
Formula10 (p = 1) Formula 11 (p = 3)		
Formula 12		
Formula 13		
Formula 14 (q = 2, X = H) Formula 15 (q = 2, X = Cl) Formula 16 (q = 3, X = Cl)		

Formulas 7 to 16

The quenchers incorporated in the compounds represented by **Formulas 7-16** are the commercially available 2-[4-(4-nitrophenylazo)-N-ethylphenylamino]ethanol (Disperse Red 1), 2-[4-(2-chloro-4-nitrophenylazo)-N-ethylphenylamino]ethanol (Disperse Red 13) and 2-[4-(dimethylamino)phenylazo]benzoic acid, identified in this invention as Red1, Red 13 and dabcyl respectively.

UV Properties of Red 13 and Dabcyl Oligonucleotide Conjugates

Figure 2 shows the absorbance properties of the red13 chromophore (**Formula 8,** without DPI_3) in comparison to dabcyl (**Formula 7**, without DPI_3 when incorporated at the 3'-end of an otherwise unmodified DNA probe. The broader absorbance (especially at long wavelengths) of the red13 chromophore is a clear advantage. Note that the 8_{max} for red13 is at 522 nm whereas the 8_{max} for dabcyl is 479 nm. The absorbance of red13 is ideal for quenching of fluorescein (emission max = 525 nm) but also overlaps with the fluorescence emission of other common laser dyes.

Quenching Properties of DPI_3 Probes with Various Quenchers and Linkers.

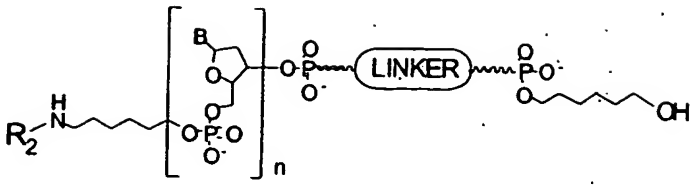
For the 10 fluorogenic probes described in **Formulas 7 to 16** the fluorescence of a standard solution of each probe was measured before and after digestion with snake venom phosphodiesterase (PDE), as described in the EXPERIMENTAL section. This PDE assay allows the quenching properties of each probe to be compared. Fluorescence of the digested probe (signal) divided by the initial fluorescence (noise) gave a signal to noise ratio (S/N), presented in **Table 1**. Larger numbers for S/N reflect more efficient fluorescent quenching (lower fluorescent background) of the intact probe.

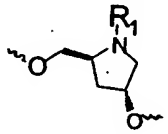
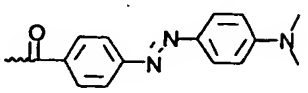
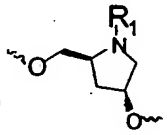
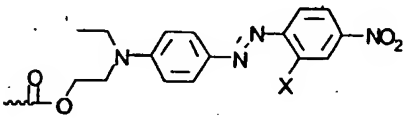
Table 1. Effect of different quenchers and linkers on fluorogenic probes shown in Formulas 7-16.

Formula # (quencher)	S/N[a] (R_2 = FAM)	S/N[a] (R_2 = TAMRA)
7 (dabcyl)	16	13
8 (red13)	21	21
9 (red1)	24	21
10 (red13)	13	33
11 (red13)	27	21
12 (dabcyl)	13	7
13 (red13)	23	21
14 (red1)	19	3
15 (red13)	24	4
16 (red13)	22	24

[a]*Signal to noise (S/N) was determined using the phosphodiesterase assay described in Example 13. The ODN sequence was 5'-gagggatgtaaaaat* **(SEQUENCE Id. No. 1)**. *The fluorophores (R_2) studied here is either 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (TAMRA).*

It is clear from the data in **Table 1** that the red13 chromophore and the closely related red1 chromophore are better quenchers for both FAM and TAMRA with a variety of linkers than dabcyl. The linker can affect quenching by the red13 chromophore. For example, **Formula 14** and **Formula 15** worked well with FAM, but had poor quenching efficiency for TAMRA. It is somewhat surprising that dabcyl worked so well, especially for the TAMRA probes. As described below, effective FRET quenching by dabcyl is a specific case for MGB probes.



	Linker Structure	R_1
Formula 17		
Formula 18 (X = Cl)		

Formulas 17 to 18

Comparison of Quenching Properties of DPI_3 Probes and Probes without DPI_3.

To further show the advantages of the red13 quencher chromophore, fluorogenic probes with a 3'-hexanol blocking group (without MGB) were compared. The structure and fluorescent properties of 13 fluorogenic probes with the same sequence were compared using the PDE assay. A red-sensitive detector was used in this study (Table 2) whereas a blue-sensitive detector was used in the study shown in **Table 1** (S/N for identical ODNs are different because different detectors have different sensitivities for the same fluorphore). The following structural variables are summarized in **Table 2**: Probe type (no-MGB vs. MGB), Quencher (dabcyl vs. Red13 vs. Red13 amide), and Reporter dye (FAM vs. TAMRA).

Table 2. Fluorescent properties of oligonucleotides with various Quenchers / Fluorophores[1].

Formula #	Probe type	Quencher	FAM (S/N)	TAMRA (S/N)
17	no- DPI_3	dabcyl	4.7	3.9
18	no- DPI_3	red13	11.6	5.8
7	DPI_3	dabcyl	23	23.5
8	DPI_3	red13	35	108
30 (*R1=2-Cl, t=v=3)*	DPI_3	red13 amide	48	97

[1]*Signal to noise (S/N) was determined using the phosphodiesterase assay described above. The ODN sequence was 5'-gagggatgtaaaaat* (**SEQUENCE Id. No. 1**). *The linker structure of the dabcyl or red13 quenchers (Q) is shown in* ***Formulas 7 and 8*** *respectively. The linker structure of the red13 amide is shown in* ***30 Reaction Scheme*** *7, R_0 is NO_2, R_1=2-Cl, R_2=R_3=R_4=-H; t=v=3.*

It is clear from the data shown in **Table 2**, that in the probes which do not contain DPI_3 the dye Red 13 quencher works better than dabcyl for both FAM and TAMRA. In DPI_3 containing probes, the dye Red13 works better in combination with FAM and much better in combination with TAMRA. Both **8** and **30** work better in DPI_3 containing probes with both fluorophores, with **30** showing the best S/N ratio for FAM Thus, it was found that the Red13 chromophore is a more efficient quencher than dabcyl for long wavelength fluorescent reporter groups. For the most commonly used fluorophore (FAM) a 2.5-fold increase in S/N was observed for standard (no-DPI_3) probes. This improved quenching by red13 is consistent with the increased spectral overlap presented in **Figure 2** and a standard FRET mechanism. The increased S/N of both **8** and **30** when incorporated into the DPI_3 probes is dramatic and

surprising. The combination of the red13 quencher and the DPI_3 resulted in a 10-fold increase in S/N for FAM quenching and a 28-fold increase in S/N for TAMRA quenching.

It is surprising and that the DPI_3 residue helps improve fluorescent quenching by the dabcyl and red13 chromophores. Without wishing to be bound by theory, it is presently postulated that the random coil conformation of the fluorogenic probe in solution is more structured in the DPI_3 probes such that the average distance between the fluorophore and quencher is closer than in probes without MGB. This closer average distance in the DPI_3 probes (tighter coil) would give rise to more efficient FRET. The exact nature of this interaction is not known, but UV spectra of the quencher and dye chromophores are not affected by the DPI_3. This is in contrast to the fluorogenic hairpin probes where the UV spectra are changed by the constrained conformation (collisional quenching).

Performance of Fluorogenic DPI_3 Probes in a "Real-Time" PCR Assay.

DPI_3 probes prepared with 5'-fluorescein and the red13 amide linker were tested in the 5'-nuclease assay to see if the hybridization properties were compatible with the linker system. As shown in **Figure 3**, both dabcyl and Red13 worked as quenchers for fluorescein in the 5'-nuclease assay when used in MGB probes. Red13 performed better than dabcyl as evidenced by the lower initial fluorescence (background) and the higher plateau after PCR. Current commercially available thermal-cycling fluorimeters can not read longer wavelength dyes in real-time PCR, but the red13 chromophore was shown to give a good S/N with TAMRA containing probes in an end point analysis after PCR.

According to another general method, the 5'-fluorophore-ODN-Q-MGB conjugates of the instant invention have improved performance in assays designed to detect DNA targets by direct hybridization. A basic description of this method is found in United States Patent No. 5,876,930, incorporated herein by reference. In this assay format, the non-hybridized

probes (quenched by FRET) become fluorescent upon forming a rigid duplex structure, thereby separating the quencher and fluorophore.

Red13 Chromophore Quenches a Broad Range of Fluorescent Reporter Groups

A series of DPI_3 probes with the red13 amide were prepared with several different fluorescent reporter groups to examine the effective range of quenching. Probes were digested with PDE as usual and showed good S/N for dyes which emit from 458 -665 nm.

Table 3. Performance of Fluorogenic DPI_3 Probes with Various Fluorophores.

Fluorophore (FL)	Ex 8 (nm)	Em 8 (nm)	S/N
coumarin	378	458	32
FAM	488	522	63
Cy3	541	565	61
TAMRA	547	582	37
resorufin	549	595	110
Cy5	641	665	36

The structure of the fluorogenic probes was FL-ODN-Q-$CDPI_3$ where Q is the red13 amide and the ODN sequence was 5'- GTC CTG ATT TTA C **(SEQUENCE Id. No. 2)**. *The fluorophores FAM, TAMRA, cy3 and cy5 were incorporated using commercially available phosphoramidite reagents. The coumarin and resorufin fluororophores were incorporated using phosphoramidites 34 and 37 which were prepared as described below.*

The fluorescent emission is well separated from FAM, as shown in the overlayed spectra in **Figure 4**. As shown in **Table 3**, the resorufin fluorescence is also quenched by the red13 chromophore. Thus the resorufin phosphoramidite has excellent properties for use in FRET probes and in combination with FAM for multicolor analysis.

As shown in **Table 3**, the coumarin fluorescence is also quenched by

the red13 chromophore. Thus, the coumarin phosphoramidite reagent can be incorporated in FRET probes and particularly in combination with FAM for multicolor analysis.

Fret-based enzyme substrates

The improved quencher molecules can be used in other FRET based assay systems. According to another general application of the invention, a quencher molecule and fluorophore are attached to an enzyme substrate, which through its catalytic action on this Q-substrate-fluorophore conjugates cleaves and separates the Q and fluorophore molecules. For example, the pentafluorophenyl activated ester **11** shown in **Reaction Scheme 3** can be used for labeling lysine residues of peptides for studying proteolytic enzymes.

EXPERIMENTAL PROCEDURES

General Experimental

All air and water sensitive reactions were carried out under a slight positive pressure of argon. Anhydrous solvents were obtained from Aldrich (Milwaukee, WI). Flash chromatography was performed on 230-400 mesh silica gel. Melting points were determined on a Mel-Temp melting point, apparatus in open capillary and are uncorrected. Elemental analysis was performed by Quantitative Technologies Inc. (Boundbrook, NJ). UV-visible absorption spectra were recorded in the 200-400-nm range on a UV-2100 (Shimadzu) or a Lambda 2 (Perkin Elmer) spectrophotometers. ^{1}H NMR spectra were run at 20EC on a Bruker WP-200 or on a Varian XL-200 spectrophotometer; chemical shifts are reported in ppm downfield from Me_4Si. Thin-layer chromatography was run on silica gel 60 F-254 (EM Reagents) aluminum-backed plates.

Example 1

2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl (2S,4R)-2-{[bis(4-methoxyphenyl)phenylmethoxy]methyl}-4-{[bis(methylethyl)amino](2-cyano-ethoxy)phosphinooxy}pyrrolidinecarboxylate (5).

2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl (5S,3R)-3-hydroxy-5-(hydroxymethyl)pyrrolidinecarboxylate (3).

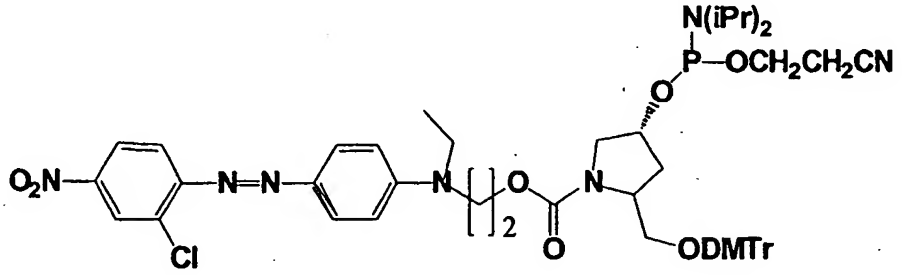
A solution of 2-[4-(2-chloro-4-nitrophenylazo)- *N*-ethylphenylamino]ethanol (Disperse Red 13, Aldrich Chemical Co., 9.0 g, 25.80 mmol) and 4-nitrophenyl chloroformate (Aldrich Chemical Co., 9.4 g, 46.61 mmol) in 90 ml of anhydrous pyridine was stirred at 70 °C for 40 min, affording intermediate **2**. Ethanol (5.0 ml) was added to the reaction solution followed by trans-hydroxyprolinol (Reed et al. Bioorg. Chem. **2:** 217-225 (1991) (42 ml of a 0.5 M solution in ethanol) and triethylamine (3.2 ml). The resultant solution was stirred for 30 min at 70 °C. The solution was evaporated to dryness and the residue was suspended in 1 liter of water and extracted with ethyl acetate (3 x 500 ml). The pooled extracts were dried over sodium sulfate, filtered and evaporated. The residue was purified by silica gel chromatography eluting with a gradient of 0-10% methanol in ethyl acetate. The pure product fractions were evaporated and precipitated from ethyl acetate - ether: 9.2 g (59%); TLC (ethyl acetate), $R_f = 0.25$. ^{1}H NMR (DMSO-d_6) δ 8.43 (1H, d, J = 2.5 Hz), 8.25 (1H, dd, J = 9.0 and 2.4 Hz), 7.86 (2H, d, J = 9.1 Hz), 7.78 (1H, d, J = 9.0 Hz), 6.96 (2H, d, J = 9.3 Hz), 4.88 (1H, m), 4.67 (1H, t, J = 5.7 Hz), 4.19 (3H, m), 3.80 (1H, m), 3.73 (2H, t, J = 5.4 Hz), 3.56 (2H, q), 3.46 (1H, t, J = 4.7 Hz), 3.27 (1H, m), 1.94 (1H, m), 1.79 (1H, m), 1.17 (3H, t, J = 6.8 Hz). Anal. Calcd for $C_{22}H_{26}ClN_5O_6 + 0.2$ H_2O: C, 53.32; H, 5.37; N, 14.13. Found: C, 53.24; H, 5.25; N, 13.99.

2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl (5S,3R)-5-{[bis(4-methoxyphenyl)phenylmethoxy]methyl}-3-

hydroxypyrrolidinecarboxylate (4).

To a solution of **3** (9.1 g, 18.53 mmol) in 130 ml of anhydrous pyridine was added 6.26 g of dimethoxytrityl chloride. The solution was stirred for 3 h. at room temperature and then poured into 300 ml of 5% sodium bicarbonate solution. The mixture was extracted with ethyl acetate (2 x 300 ml) and the combined extracts were dried over sodium sulfate, filtered and evaporated. The residue was purified by silica gel chromatography eluting with a gradient of 20-0% hexane in ethyl acetate followed by a gradient of 0-2% methanol in ethyl acetate. The chromatography eluent also contained 1% triethylamine. The pure product fractions were combined affording an amorphous solid: 12.66 g (86%); TLC (ethyl acetate), $R_f = 0.44$. ^{1}H NMR (DMSO-d_6) δ 8.45 (1H, s), 8.26 (1H, d, J = 8.9 Hz), 7.82 (3H, m), 7.27 (4H, m), 7.16 (5H, m), 6.95-6.79 (6H, m), 4.95 (1H, m), 4.32 (1H, m), 4.14 (1H, m), 3.99 (2H, m), 3.73 (1H, m), 3.69 (6H, s), 3.56 (1H, m), 3.40-3.30 (2H, m), 3.14 (1H, m), 2.10-1.82 (2H, m), 1.16 (3H, m), 1.06 (3H, t, J = 6.5 Hz). Anal. Calcd for $C_{43}H_{44}ClN_5O_8 + 0.2\ H_2O$: C, 64.73; H, 5.61; N, 8.78. Found: C, 65.08; H, 5.70; N, 8.31.

2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl (2S,4R)-2-{[bis(4-methoxyphenyl)phenylmethoxy]methyl}-4-{[bis(methylethyl)amino](2-cyano-ethoxy)phosphinooxy}pyrrolidinecarboxylate (5).



5

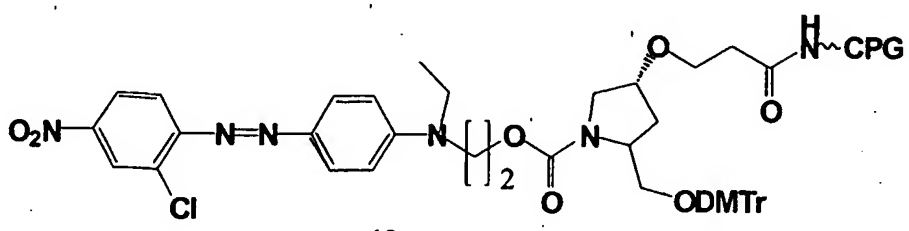
To a solution of **4** (12.63 g, 15.91 mmol) dissolved in 440 ml of anhydrous methylene chloride, containing 8.0 ml of N,N-diisopropylethylamine, was added 5.94 ml of 2-cyanoethyl

diisopropylchlorophosphoramidite. The solution was stirred 30 min under argon at room temperature. The reaction mixture was treated with 10 ml of methanol and poured into 400 ml of 5% sodium bicarbonate solution. The organic phase was dried over sodium sulfate and evaporated. The residue was purified by silica gel chromatography eluting with a gradient of 40-20% hexane in ethyl acetate (2% triethylamine). The pure product fractions were evaporated affording an amorphous solid: 14.75 g (93% yield). ^{31}P NMR (DMSO-d_6) δ 146.93 (singlet). Anal. Calcd for $C_{52}H_{61}ClN_7O_9$ + 1.0 H_2O: C, 61.68; H, 6.27; N, 9.68. Found: C, 61.44; H, 6.47; N, 9.35.

Example 2

RED 13-pyrrolidine-DMTr-CPG 12

Synthesis of pentafluorophenyl ester (11) and RED 13_pyrrolidine_DMTr_CPG (12) Reactions Scheme 3.



12

The pentafluorophenyl ester (**11**) is synthesized by the same method used for the synthesis of Compound **22** as described in Example 4 and **Reaction Scheme 5**.

RED 13-pyrrolidine-DMTr-CPG (12)

10 g of LCAA_CPG was combined with 5 ml of a 0.3 M solution of 11 in DMF and agitated gently overnight, when it was filtered and washed with 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether. Traces of ether were removed in vacuo (oil pump). Unreacted amino groups were acetylated by treating the CPG with 40 mL of dry pyridine and 5 mL of acetic anhydride. After swirling for 1.5 h, the CPG was filtered and washed

with 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether. Traces of ether were removed in vacuo (oil pump). The CPG was analyzed for MMT loading by treating 3_5 mg of CPG in 25 mL of 1:1 / 70% perchloric acid:methanol. The absorbance of the released MMT cation was recorded at 472 nm and loading level was adjusted to be between 30_40 mmol / g of CPG using the equation:

MMT loading (mmol / g) = A472 x volume (in mL) x 14.3 , wt of CPG (mg)

Example 3

2-(4-Nitrophenyl)ethyl 3-(pyrrolo[4,5-e]indolin-7-ylcarbonyl)pyrrolo[4,5-e]indoline-7-carboxylate (17). (Reaction Scheme 4)

2-(4-Nitrophenyl)ethyl 3-[(tert-butyl)oxycarbonyl]pyrrolo[4,5-e]indoline-7-carboxylate (14).

Ten grams (33.1 mmol) of 3-[(tert-butyl)oxycarbonyl]pyrrolo[3,2-e]indoline-7-carboxylic acid (Boger, D.L., Coleman, R.S., Invergo, B.J. (1987) *J. Org. Chem. 52*, 1521.), well dried, are placed into an argon filled flask, and 84 mL of THF and 10.4 mL (66.2 mmol) of diethyl azodicarboxylate (DEAD) are added. Then a dropping funnel is placed atop the flask (flushed with argon) and a water bath (to cool the flask) is placed under it. A solution of 17.3 g (66 mmol) of triphenylphosphine and 6.64 g (39.7 mmol) of 2-(p-nitrophenyl) ethanol in 160 mL of ethyl ether is made. This solution is added to the dropping funnel, and then to the reaction flask, dropwise, with stirring. The reaction is allowed to proceed for an hour, at which time, a TLC analysis is done (2:1 hexanes/ethyl acetate) examined by UV (254 nm) to determine whether the reaction is complete. If it is complete, then the baseline spot (bluish) will disappear and the product, with an R_f of 0.55, will appear as a dark spot. Often, especially if the reactants are not entirely dry, another portion of triphenylphosphine and DEAD are required. If so, a tenth of the original amounts is usually sufficient, i.e., 1.73 g of

triphenylphosphine and 1.04 mL of DEAD. These can be added neat to the stirred solution. Allow to react another hour, after which another TLC analysis usually reveals complete reaction. The product usually precipitates out in part; this is collected bt filtration and washed with methanol, then recrystallized by dissolving in a minimum amount (typically, 80 - 100 ml) of warm acetone and adding four times that volume of warm methanol. Cool to 4°C for several hours or perhaps overnight. The supernatant from the original precipitation is saved and evaporated to a syrup or until dry. It too is dissolved in a minimum amount of warm acetone; typically about 100 - 120 mL of warm acetone. The total amount or acetone used for the two recrystallizations is usually approximately 200 mL. As before, an amount of warm methanol equal to four times the amount of acetone is added. The solution is cooled; crystallization begins almost at once but is allowed to continue several hours to overnight. The recrystallizations are quite efficient, but the product from the reaction supernatant is usually not quite as pure and is purified by recrystallization. The yield is approximately 85%. (mp 191-193°C) ^{1}H NMR (DMSO-*d6*) δ 11.83 (s, 1H), 8.18 (d, J=8.5 Hz, 2H), 7.84 (br s, 1H), 7.64 (d, J=8.5 Hz, 2H), 7.25 (d, J=8.8 Hz), 6.96 (s, 1H), 4.56 (t, J=6 Hz, 2H), 4.00 (t, J=8.8 Hz, 2H), 3.21 (m, 4H), 1.51 (s, 9H). Combustion Analysis: Found: C, 63.16%; H, 5.56%; N, 9.45%. Calculated for 0.4 mole added water: C, 62.8%; H, 5.7%; N, 9.16%.

2-(4-Nitrophenyl)ethyl pyrrolo[4,5-e]indoline-7-carboxylate (15).

Two grams (4.43 mmol) of **14** are weighed into a round bottomed flask. Then, in a fume hood, 25 mL (325 mmol) of trifluroacetic acid is added, and the flask is capped and stirred. The solid dissolves in about a minute. The mixture is stirred for 1 hour, at which time deprotection will be done (HPLC can be used as a check). The acid is evaporated on a rotary evaporator (use a trap) and the product is dissolved in 100 mL of methylene chloride. This is extracted twice with 100 mL of half to 2/3 saturated sodium

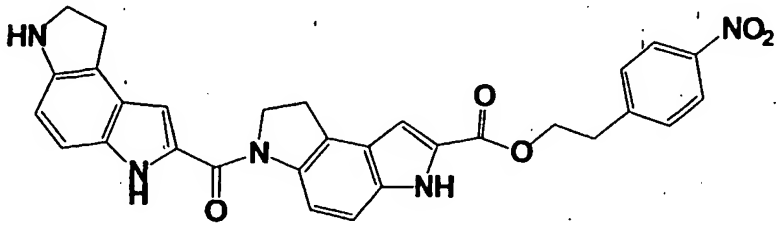
bicarbonate solution. The aqueous layers are back-extracted once with ~50 mL of methylene chloride and this is combined with the rest. The organic layer is dried over sodium sulfate twice and evaporated to give a brown solid. If desired, this material can be recrystallized by diluting a very concentrated solution in methylene chloride with methanol and cooling. Yields approaching 100% are usually obtained. mp 192-194°C. ^{1}H NMR (DMSO-*d6*) δ 11.51 (s, 1H), 8.18 (d, J=8.5 Hz, 2H), 7.63 (d, J=8.5 Hz, 2H), 7.11 (d, J=8.5 1H), 6.80 (s, 1H), 6.70 (d, J=8.5 Hz), 5.03 (br s, 1H), 4.54 (t, J=6.4 Hz, 2H), 3.46 (t, J=8.6 Hz, 2H), 3.19 (m, 2H), 3.04 (t, J=8.6 Hz, 2H). Combustion Analysis: Calculated for $C_{19}H_{17}N_3O_4$: C,64.94%; H, 4.88%; N, 11.96%. Found: C, 65.50%; H, 4.70%; N, 11.64%

2-(4-Nitrophenyl)ethyl 3-({3-[(tert-butyl)oxycarbonyl]pyrrolo[4,5-e]indolin-7-yl}carbonyl)pyrrolo[4,5-e]indoline-7-carboxylate (16).

3.09 grams (8.8 mmol) of **15** is mixed with 2.66 grams (8.8 mmol) of **13** (Boger, D.L., Coleman, R.S., Invergo, B.J. (1987) *J. Org. Chem. 52*, 1521.), and 46 mL of DMF is added. Then 3.85 grams (8.77 mmol) of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride are added. The mixture is stirred for about three hours. The mixture is initially homogeneous, but as the stirring proceeds, a precipitate of the product forms. The solvent DMF is evaporated under a high vacuum, and about 100 mL of methanol is added. The mixture is swirled and filtered in a sintered glass funnel, then thoroughly washed with 3 X 50 mL portions of methanol. Then it is dried in vacuo. Yields usually approach 100 percent. mp: 132E-134°C. ^{1}H NMR (DMSO-*d6*) *: 11.93 (s, NH, 1H), 11.62 (s, NH, 1H), 8.30 (br s, aromatic proton, 1H), 8.27 (br s, aromatic proton, 1H), 8.19 (d, aromatic protons, J=8.3 Hz, 2H), 7.65 (d, aromatic protons, J=8.3 Hz, 2H), 7.34 (d, J=9 Hz, aromatic proton, 1H), 7.29 (d, J=9 Hz, aromatic proton, 1H), 7.07 (s, aromatic proton, 1H), 6.98 (s, aromatic proton, 1H), 4.60 (m, aliphatic protons, 4H), 4.02 (t, J=8.5 Hz, aliphatic protons, 2H), 3.40 (t, J=8 Hz,

aliphatic protons, 2H), 3.24 (m, aliphatic protons, 4H), 1.52 (s, 3xCH_3, 9H). Combustion Analysis: Calculated: C, 66.13%; H, 5.23%; N, 11.02%. Found: C, 65.94%; H, 5.19%; N, 11.07%.

2-(4-Nitrophenyl)ethyl 3-(pyrrolo[4,5-e]indolin-7-ylcarbonyl)pyrrolo[4,5-e]indoline-7-carboxylate (17).



17

5 grams of **16** are placed in a flask. 100 mL of trifluoroacetic acid is added, and the mixture is stirred. After an hour, the acid is evaporated on a rotary evaporator and 100 mL saturated sodium bicarbonate solution and 100 mL of water are added. The mixture is agitated or sonicated for ~1/2 hours, then filtered and washed with water and then methanol, and dried in vacuo. The material may be recrystallized. It is dissolved in a minimum amount of warm DMF, and then approximately a threefold portion of methanol is added and the solution is sonicated a few minutes. A cream to brown material crystallizes out. This is washed with methanol, and dried in vacuo. The yield approaches theoretical values. ^{1}H NMR (DMSO-*d6*) δ 11.96 (s, NH, 1H), 11.71 (s, NH, 1H), 8.30 (br s, aromatic proton, 1H), 8.27 (br s, aromatic proton, 1H), 8.19 (d, aromatic protons, J=8.5 Hz, 2H), 7.66 (d, aromatic protons, J=8.3 Hz, 2H), 7.34 (m, aromatic protons, 2H), 7.08 (s, aromatic proton, 1H), 7.03 (s, aromatic proton, 1H), 4.60 (m, aliphatic protons, 4H), 3.68 (t, J=8 Hz, aliphatic protons, 2H), 3.40 (t, J=8 Hz, aliphatic protons, 2H), 3.24 (m, aliphatic protons, 4H). Combustion Analysis: Found: C, 63.55 %; H, 4.42 %; N, 11.95 %. Calculated, for ½ mole sodium bicarbonate contaminant: C, 63.43 %; H, 4.45 %; N, 12.13 %.

Example 4

2,3,4,5,6-pentafluorophenyl 3-[4-({3-[bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]pyrrolo[4,5-e]indoline-7-carboxylate (24) (Reaction Scheme 5)

Ethyl 4-[(3-hydroxypropyl)phenylamino]butanoate (19)

A mixture of 3-(phenylamino)propan-1-ol (Huang, Yande; Arif, Atta M.; Bentrude, Weseley G.; *J.Org.Chem.*; 58(23) 1993; 6235-6246) (65.6 g, 0.43 mol), ethyl 4-bromobutyrate (104.5 g, 0.54 mol) and 100 mL of ethyldiisopropylamine is stirred at 100°C for 1 h. The reaction is cooled to room temperature and partitioned between water 400 mL and ethyl acetate (500 mL). The organic layer is washed with saturated $NaHCO_3$, brine and dried over Na_2SO_4. The oil obtained after concentration is chromatographed on silica eluting with 10% EtOH/$CHCl_3$. Concentration of the appropriate fractions affords 115 g (100%) of the desired product as a colorless, viscous oil. ^1H NMR ($CDCl_3$) δ 7.23 (m, 2H), 6.72 (m, 3H), 4.14 (q, J=7 Hz, 2H), 3.72 (t, J=6Hz, 2H), 3.43 (t, 7 Hz, 2H), 3.34 (t, 7 Hz, 2H), 2.35 (t, 7 Hz), 1.88 (m, 4H), 1.26 (t, 7 Hz, 3H).

Ethyl 4-({4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}(3-hydroxypropyl)amino)butanoate (20)

2-Chloro-4-nitroaniline 2.5 g (10 mmol) is placed into a 125 mL flask and 6 mL of water is added. Agitation and sonication partially dissolves the yellow chloronitroaniline. Then the stirred solution is cooled with ice in a fume hood and 15.8 mL of concentrated (~12 M) HCl is added. Most of the yellow material dissolves at this point. The flask is fitted with a dropping funnel, and a solution of 1.51 g (21.9 mmol) sodium nitrite in 3-4 mL of water is added to the dropping funnel and slowly added to the solution in the flask with stirring, over about 20 minutes. When this is complete, 0.6 g (~21

mmol) of urea is added followed by 2.73 g of ethyl 4-[(3-hydroxypropyl)phenylamino]butanoate as a solution in 8.2 mL acetic acid. After a minute 20 g of sodium acetate in ~50 mL of water is added. The mixture is allowed to stir for an hour at room temperature. Most of the product is separated as an emulsion. The mixture is partitioned between ethyl acetate and water. The organic layer is washed with $NaHCO_3$ (3x 50 ml), brine and dried over anhydrous sodium sulfate. Then the organic solvents are evaporated to a syrup. The crude product is chromatographed on silica gel (1.5 x 20 inches) eluting with 50% ethyl acetate/hexane. The appropriate fractions are collected, combined, evaporated (30-40 degrees), and dried in a vacuum. The product is a dark oil. The yield is approximately 68-70%.^{1}H NMR (DMSO-*d6*) δ 8.42 (d, J=2.5 Hz, aromatic proton, 1H), 8.24 (dd, J_1=9 Hz, J_2=2.5 Hz, aromatic proton, 1H), 7.86 (d, J=9Hz, 2H), 7.77 (d, J=9 Hz, 1H), 6.92 (d, J=9 Hz, aromatic protons, 2H), 4.67 (t, J=6 Hz, OH, 1H), 4.07 (q, J=7 Hz, CH_2O, 2H), 3.5 (m, aliphatic protons, 6H), 2.40 (t, J=7 Hz, aliphatic protons, 2H), 1.84 (m, aliphatic protons, 2H), 1.72 (m, aliphatic protons, 2H), 1.18 (t, J=7 Hz, CH_3, 3H).

4-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}(3-hydroxypropyl)amino)butanoic acid.

To a stirred solution of **20** (4.48 g, 10 mmole) in 40 mL of THF added 40 mL of ethanol followed by a solution of KOH (0.84 g,15 mmol) in 20 mL of water and 20 mL of ethanol. The mixture is stirred overnight and concentrated. The residue suspended in 125 mL of water, treated with 2.6 mL (~ 3 eqv.) of acetic acid, and cooled to 4°C. The resulting solid is filtered off, washed with water, and dried. Yield is quantitative. ^{1}H NMR (DMSO-*d6*) δ 8.42 (d, J=2.5 Hz, aromatic proton, 1H), 8.23 (dd, J_1=9 Hz, J_2=2.5 Hz, aromatic proton, 1H), 7.82 (d, J=9Hz, 2H), 7.90 (d, J=9Hz, 1H), 7.03 (d, J=9 Hz, aromatic protons, 2H), 4.8 (br s, OH, 1H), 3.5 (m, aliphatic protons, 6H), 1.86 (t, J=6 Hz, aliphatic protons, 2H), 1.72 (m, aliphatic protons, 4H).

4-({3-[Bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoic acid (21)

4.21 g (10 mmol) of the acid from the previous step is placed into a 250 mL round bottom flask. Dry pyridine (50-100 ml) is added and evaporated (30-40 degrees) with a rotary evaporator. The process is repeated once or twice to remove all traces of water. Dry pyridine (80 mL) is added to the contents of the flask. Then 4.07 g (12 mmol) of dimethoxytrityl chloride is added. After being stirred for 1 h pyridine is evaporated and the resulting syrup is dissolved in a few milliliters of 18:1:1 methylene chloride/methanol/ triethylamine. A silica gel column (~1.5" x 20") is prepared with an eluent of 18:1:1 methylene chloride/methanol/ triethylamine and the product is run through the column, collecting and combining the appropriate fractions. After the solvents are removed by evaporation the resulting amorphous solid contains some triethylammonium salts in addition to the desired product. The impurity does not interfere with the next step and the product is used without additional purification.

2,3,4,5,6-Pentafluorophenyl 4-({3-[bis(4-methoxyphenyl)phenylmethoxy]-propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoate (22).

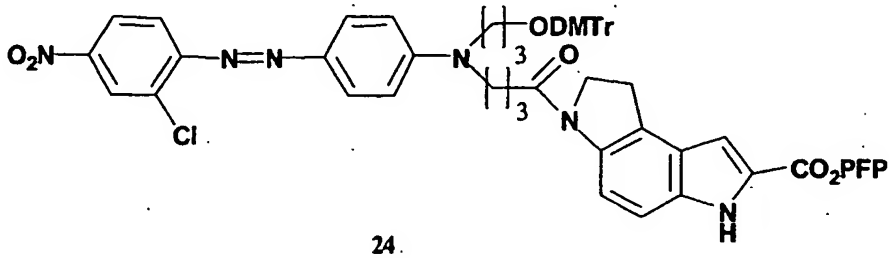
To the flask containing **21** (10 mmol) is added 7 mL of triethylamine followed by 100 mL of methylene chloride, 2.05 mL of pentafluorophenyl trifluoroacetate (PFP-TFA) is then added. The solution is stirred for half an hour. At the end of this time, the reaction is usually complete. (TLC: 2:1 hexane/ethyl acetate). The solvent is removed on the rotary evaporator to give a syrup which is chromatographed on silica eluting with 1:3 ethyl acetate/hexane. Appropriate fractions are collected, combined, evaporated and dried under vacuum. The yield is 41%. ^{1}H NMR (DMSO-*d6*) δ 8.43 (d, J=2.5 Hz, aromatic proton, 1H), 8.24 (dd, J_1=9 Hz, J_2=2.5 Hz, aromatic proton, 1H), 7.83 (d, J=9 Hz, aromatic proton, 1H), 7.78 (d, J=9 Hz, aromatic

proton, 1H), 7.42-7.15 (m, aromatic protons, 10H), 7.07 (m, aromatic protons, 2H), 7.00-6.80 (m, aromatic protons, 4H), 3.72 (s, 2xCH_3, 6H), 3.56 (m, aliphatic protons, 2H), 3.48 (t, J=6.3 Hz, aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H), 2.89 (t, J=7 Hz, aliphatic protons, 2H), 1.95 (m, aliphatic protons, 2H), 1.86 (m, aliphatic protons, 2H).

Methyl 3-[4-({3-[bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]pyrrolo[4,5-e]indoline-7-carboxylate (23).

To a solution of **22** (3.0 g, 3.37 mmol) in 15 mL anhydrous DMF is added triethylamine (0.75 mL) followed by methyl pyrrolo[4,5-e]indoline-7-carboxylate (Boger, D.L., Coleman, R.S., Invergo, B.J. (1987) *J. Org. Chem. 52*, 1521.) (0.8 g, 3.7 mmol). The resultant solution is stored at room temperature for 20 h. The reaction is analyzed by HPLC to confirm its completeness. DMF is removed on a rotary evaporator equipped with an oil pump. The residue, dark syrup is suspended in 50% ethylacetate/hexanes (~25 mL). The mixture is sonicated to initiate the crystallization. The crystals are stirred for 15 min, collected by filtration on a sintered glass funnel, washed with methanol (2x30 mL) and dried under vacuum. The yield of the desired product is 2.7 g (87%) as a deep-purple solid. ^{1}H NMR (DMSO-*d6*) δ 11.93 (d, J=1.7 Hz, indole NH, 1H), 8.43 (d, J=2.5 Hz, aromatic proton, 1H), 8.3-8.2 (m, aromatic protons, 2H), 7.85-7.75 (m, aromatic protons, 3H), 7.45-7.18 (m, aromatic protons, 10 H), 7.05 (d, J=1.8 Hz, aromatic proton, 1H), 6.97 (d, J=9 Hz, aromatic protons, 2H), 6.87 (d, J=9 Hz, aromatic protons, 4H), 4.12 (t, J=8 Hz, aliphatic protons, 2H), 3.87 (s, ester CH_3, 3H), 3.71 (s, CH_3, 6H), 3.60 (br t, aliphatic protons, 2H), 3.45 (br t, aliphatic protons, 2H), 3.29 (br t, aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H), 2.5 (br t, obscured by DMSO signal, aliphatic protons, 2H), 1.88 (br m, aliphatic protons, 4H).

2,3,4,5,6-pentafluorophenyl 3-[4-({3-[bis(4-methoxyphenyl)phenylmethoxy]-propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]-pyrrolo[4,5-e]indoline-7-carboxylate (24)



24

1. Hydrolysis of the methyl ester

To a solution of **23** (2.67 g, 2.9 mmol) in 25 mL THF are added methanol (25 mL) and 5% LiOH, monohydrate in H_2O (10 mL). The resultant suspension is stirred at 50°C (bath temperature) for 90 min. by which time a clear solution is obtained. TLC analysis shows no starting material. Solvent is removed under vacuum and the product is partitioned between CH_2Cl_2 and cold 10% citric acid. The organic phase is neutralized with triethylamine, dried over Na_2SO_4 and concentrated. The resultant product (amorphous solid) is dried in high vacuum for at least 3 h and used in the next step without additional purification.

2. PFP ester preparation

The product obtained in the previous step is dissolved in 100 mL anhydrous DMF. Triethylamine (2 mL) is added followed by PFP-TFA (2 mL, 4.4 mmol). The reaction is stirred for 30 min and analyzed by HPLC. No starting material, free acid should be observed. DMF is evaporated and the residue, deep purple syrup is suspended in 100 mL MeOH. After stirring for 30 min, a dark precipitate is formed which is collected by filtration on a sintered glass funnel, washed with methanol (3x20 mL) and dried under vacuum (15 - 30 h). This procedure yields 2.7 g (94%) of the desired product as a purple solid. ^{1}H NMR (DMSO-*d6*) δ 12.45 (d, J=1.8 Hz, indole NH, 1H), 8.43 (d, J=2.5 Hz, aromatic proton, 1H), 8.38 (d, J=9 Hz, aromatic proton, 1H), 8.24 (dd, J_1=9 Hz, J_2=2.5 Hz, aromatic proton, 1H), 7.85-7.75 (m, aromatic protons, 3H), 7.52-7.18 (m, aromatic protons, 11 H), 6.97 (d,

J=9 Hz, aromatic protons, 2H), 6.88 (d, J=9 Hz, aromatic protons, 4H), 4.16 (t, J=8.5 Hz, aliphatic protons, 2H), 3.71 (s, CH_3, 6H), 3.61 (br t, aliphatic protons, 2H), 3.47 (br t, aliphatic protons, 2H), 3.32 (br t, aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H), 2.5 (br t, obscured by DMSO signal, aliphatic protons, 2H), 1.88 (br m, aliphatic protons, 4H).

Example 5

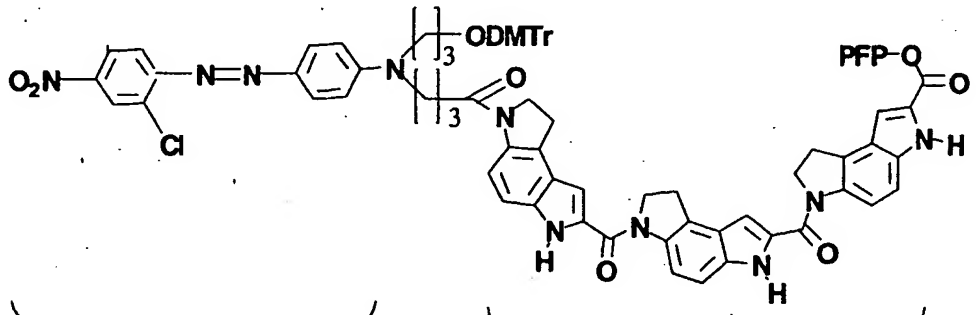
2,3,4,5,6-Pentafluorophenyl 3-{[3-({3-[4-({3-[bis(4-methoxyphenyl)-phenylmethoxy]propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}-amino)butanoyl]pyrrolo[4,5-e]indolin-7-yl}carbonyl)pyrrolo[4,5-e]indolin-7-yl]carbonyl}pyrrolo[4,5-e]indoline-7-carboxylate (25a). where R_1= 2-Cl and t=v=3 (Reaction Scheme 6)

2-(4-Nitrophenyl)ethyl 3-{[3-({3-[4-({3-[bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]pyrrolo[4,5-e]indolin-7-yl}carbonyl)pyrrolo[4,5-e]indolin-7-yl]carbonyl}pyrrolo[4,5-e]indoline-7-carboxylate (25).

Into a 100 mL round bottom flask is weighed out 1.31 g (1.22 mmol) of **24**. This is dissolved in 25 mL of dimethylformamide. Then 0.81 mL of triethylamine is added, and finally 0.623 g (1.162 mmol) of **17**. The reaction mixture is left overnight, then the solution is concentrated to ~10 mL and the resultant precipitate is filtered off, using a sintered glass filter funnel. The solid is washed with generous volumes of methanol (stirring the sludge in the filter with the methanol before applying the vacuum) several times and ether. When the effluent is clear and essentially colorless, the deep violet precipitate is dried in vacuo to afford 1.5 g (90%) of the desired product. ^{1}H NMR (DMSO-*d6*) δ 11.96 (s, indole NH, 1H), 11.76 (s, indole NH, 1H), 11.69 (s, indole NH, 1H), 8.43 (d, J-2.4 Hz, aromatic proton, 1H), 8.35-8.20 (m, aromatic protons, 4H), 8.19 (d, J=9 Hz, aromatic protons, 2H), 7.85-7.75 (m,

aromatic protons, 3H), 7.66 (d, J=9Hz, aromatic protons, 2H), 7.45-7.18 (m, aromatic protons, 12H), 7.10 (s, aromatic proton, 1H), 7.01 (s, aromatic proton, 1H), 6.99 (m, aromatic protons, 3H), 6.88 (d, J=9 Hz, aromatic protons, 4H), 4.61 (m, aliphatic protons, 6H), 4.14 (t, J=8.5 Hz, aliphatic protons, 2H), 3.71 (s, 2xCH_3O, 6H), 3.59 (m, aliphatic protons, 2H), 3.43 (m, aliphatic protons, 6H), 3.34 (m, obscured by water signal, aliphatic protons, 2H), 3.22 (m, aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H), 2.5 (t, obscured by DMSO signal, $COCH_2$-, 2H), 1.89 (br m, aliphatic protons, 4H). Analysis: Calculated: C, 68.27 %; H, 4.95 %; N, 10.81%. Found: C, 68.08 %; H, 4.98 %; N, 10.63 %.

2,3,4,5,6-Pentafluorophenyl 3-{[3-({3-[4-({3-[bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]pyrrolo[4,5-e]indolin-7-yl}carbonyl)pyrrolo[4,5-e]indolin-7-yl]carbonyl}pyrrolo[4,5-e]indoline-7-carboxylate (25a).



Into a flask is placed 1.0 g (0.73 mmol) of the product from the previous step, 40 mL of THF, and 2.46 g of DBU. The mixture is stirred at 50 degrees for 4 hours, then removed from the heat and evaporated to 15 to 20 ml. About 40 mL of methanol is added to the product and the mixture is agitated and sonicated. Then the precipitate is filtered off with a sintered glass funnel and washed with 40-60 mL of additional methanol, followed by a similar amount of ethyl ether, each time stirring the material in the filter prior

to applying the vacuum so that the effluent soon becomes clear. The product is dried in vacuo for an hour or two before it is used in the next step. The material is dissolved in 20 mL of DMF in a 100 mL flask and stirred to dissolve. Then 0.6 mL (4.3 mmol) of triethylamine is added, followed by 0.6 mL of PFP-TFA. The reaction mixture is stirred under argon overnight, and then evaporated to a gum and a ~10 mL of DMF is added, followed by ~ 80 mL of methanol. This mixture is swirled and sonicated, and then the product, which precipitates out, is filtered off and dried in vacuo. Yield is 85-90%. ^{1}H NMR (DMSO-*d6*) δ 12.01 (s, indole NH, 1H), 11.76 (s, indole NH, 1H), 11.69 (s, indole NH, 1H), 8.43 (d, J-2.4 Hz, aromatic proton, 1H), 8.40 (br s, aromatic proton, 1H), 8.35-8.20 (m, aromatic protons, 3H), 7.85-7.75 (m, aromatic protons, 3H), 7.59 (d, J=1.2 Hz, aromatic proton, 1H), 7.45-7.18 (m, aromatic protons, 12H), 7.13 (s, aromatic proton, 1H), 6.99 (m, aromatic protons, 3H), 6.88 (d, J=9 Hz, aromatic protons, 4H), 4.66 (m, aliphatic protons, 4H), 4.14 (t, J=8.5 Hz, aliphatic protons, 2H), 3.71 (s, 2xCH_3O, 6H), 3.59 (m, aliphatic protons, 2H), 3.43 (m, aliphatic protons, 6H), 3.34 (m, obscured by water signal, aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H), 2.5 (t, obscured by DMSO signal, $COCH_2$-, 2H), 1.89 (br m, aliphatic protons, 4H). Analysis: Found: C, 63.58 %; H, 4.13 %; N, 9.53 %. Calculated, for 2.3 moles of water: C, 63.97 %; H, 4.21 %; N, 9.44 %.

Example 6

DMTrO-Red 13-amide-CDPI$_3$-CPG (29) (Reaction Scheme 7)

3-[(4-Methoxyphenyl)diphenylamino]propan-1-ol (26).

4 g (53 mmol) of 3-aminopropanol was dissolved by stirring in 50 mL of methylene chloride in an oven dried 250 mL round bottom flask. This solution was stoppered and set aside. 7.7 g (24.9 mmol) of monomethoxytrityl chloride (MMT-Cl, Aldrich reagent grade) was dissolved in another 50 ml of methylene chloride. An oven dried dropping funnel was fitted to the flask and the MMT-Cl solution was added to the funnel. The

MMT-Cl solution was then added to the solution in the flask over ~10 min (some heat develops). After an hour the reaction was analyzed by TLC (1:1 v/v hexanes/ethyl acetate, R_f 0.4) and found to be complete. Visualization of TLC spots by ninhydrin spray / heat showed a trace of (faster moving) bis-MMT side product. The reaction mixture was added to 200 mL of water standing over 200 mL of methylene chloride in a separatory funnel. The mixture was shaken and separated into layers; the aqueous layer was discarded and the organic layer was washed with an additional 200 mL of water. The organic layer was dried over 10-20 g of sodium sulfate and evaporated to give ~ 7 g of the tritylated amine as a pale yellow syrup. This compound did not require further purification and was dried overnight. After several days the syrup solidified. The product was recrystallized from ether-hexanes to give 4.6 g (53% yield) of **26** as a white solid (mp = 89.5 - 90.5 EC). Anal. calcd for $C_{23}H_{25}NO_2$: C, 79.51; H, 7.25; N, 4.03. Found: C, 79.48; H, 7.18; N, 3.98.

2-[({3-[(4-Methoxyphenyl)diphenylamino]propyl}oxycarbonyl)methoxy]-acetic acid, triethylammonium salt (27).

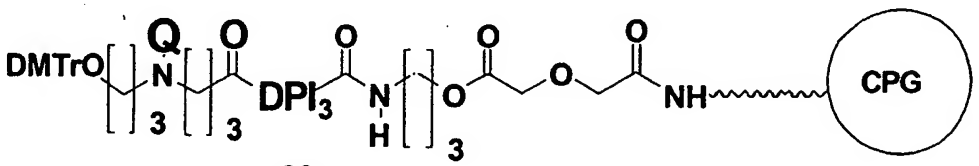
2.72g (7.83 mmol) of the alcohol (**26**) was dissolved in 20 mL of methylene chloride with 1.3 mL (9.4 mmol) of triethylamine and 1.1 g (9.5 mmol) of glycolic anhydride. The mixture was stirred for 2 h (became homogeneous). TLC showed clean reaction (Rf = 0.35 in 9:1 / methylene chloride:methanol). The solvents were removed by evaporation and the residue was chromatographed on a 1.5 x 18 inch silica gel column packed with 93% methylene chloride, 5% methanol, and 2% triethylamine. The fractions containing product were combined and solvent was removed by evaporation. Co-evaporation with dry DMF ensured removal of traces of water and of residual volatile solvents. Yield of the colorless syrup (**27**) was assumed to be 100%. The syrup was dissolved in dry DMF to give a final volume of 23.4 mL (~ 0.33 M solution).

Synthesis of N-MMT diglycolate CPG (28).

10 g of LCAA-CPG was combined with 5 mL of a 0.33 M solution of **27** in DMF (1.66 mmol) in a 100 mL round bottom flask. A solution of 2.5 mL of diisopropylethylamine, 0.11 g (0.8 mmol) of HOBT and 0.63 g (1.66 mmol) of HBTU was prepared and added to the CPG. The mixture was stoppered and swirled for 16 h on an orbital shaker (150 rpm). The CPG was filtered on a medium porosity sintered glass funnel and washed with 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether. Traces of ether were removed in vacuo (oil pump). Unreacted amino groups were acetylated by treating the CPG with 40 mL of dry pyridine and 5 mL of acetic anhydride. After swirling for 1.5 h, the CPG was filtered and washed with 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether. Traces of ether were removed in vacuo (oil pump). The CPG was analyzed for MMT loading by treating 3-5 mg of CPG in 25 mL of 1:1 / 70% perchloric acid:methanol. The absorbance of the released MMT cation was recorded at 472 nm and loading level was calculated to be 95.7 :mol / g of CPG using the equation:

MMT loading (:mol / g) = A_{472} x volume (in mL) x 14.3 ÷ wt of CPG (mg)

Synthesis of CPG 29.



29

4 g of N-MMT diglycolate CPG (**28**) was weighed into a medium porosity sintered glass funnel. The CPG was detritylated by treating with 25 mL of 3% TCA / DCM. After stirring briefly with a spatula, the mixture reacted for 5 min before filtering (turned yellow). The process was repeated 4 times until the filtrate was colorless. The CPG was washed with 4x 40 mL of

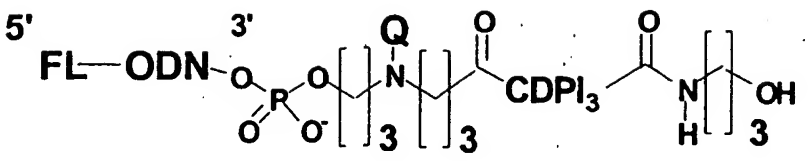
methylene chloride. The filtrate was discarded to organic waste, and the CPG was neutralized by treatment with 40 mL of 20% triethylamine in acetonitrile. After briefly stirring with a spatula, the mixture was filtered and washed with 2x 40 mL of acetonitrile, and 2x 40 mL of ether. Traces of ether were removed in vacuo (oil pump). The de-tritylated CPG was used immediately for the following immobilization reaction.

0.259 g (180 :mol) of **25a** was shaken with 12 mL of dry DMSO in a 15 mL polypropylene tube. After 15 min, the dark purple solution was added to 4 g of detritylated diglycolate CPG (in a 50 mL round bottom flask). This corresponds to an offering ratio of 45 :mol PFP ester per gram of CPG. An additional 5 mL of DMSO was added to the polypropylene tube to dissolve residual PFP ester and the solution was added to the CPG. 2 mL of triethylamine was added and the mixture was stoppered and swirled on an orbital mixer for 14 h. The CPG was filtered and washed with 2x 50 mL of DMSO, 2x 50 mL of acetonitrile, and 2x 50 mL of ether. Traces of ether were removed in vacuo (oil pump). Unreacted amino groups were acetylated by treating the CPG with 10 mL of dry pyridine and 3 mL of acetic anhydride. After swirling for 6 h, the CPG was filtered and washed with 2x 50 mL of DMF, 2x 50 mL of acetonitrile, and 2x 50 mL of ether. Traces of ether were removed in vacuo (oil pump). The CPG was analyzed for DMT loading by treating 3-5 mg of CPG in 25 mL of 1:1 / 70% perchloric acid:methanol. The absorbance of the released DMT cation was recorded at 498 nm and loading level was calculated to be 45 :mol / g of CPG using the equation:

DMT loading (:mol / g) = A_{498} x volume (in mL) x 14.3 ÷ wt of CPG (mg)

Example 7

Synthesis of FL-ODN-Red 13-amide-$CDPI_3$ (30).



30

The oligonucleotides were synthesized on the CPG 29 using standard phosphoramidite coupling chemistry except that the standard 0.1 I_2 oxidizing solution was diluted to 0.01-0.015 to avoid iodination of the MGB moiety. FAM and TET were incorporated at the 5'end using the corresponding phosphoramidites available from Glen Research.

Example 8

4-{[N-(6-{[Bis(methylethyl)amino](2-cyanoethoxy)phosphinooxy}hexyl)carbamoyl]methyl}-2-oxo-2H-chromen-7-yl 2,2-dimethylpropanoate (34a).

N-(6-Hydroxyhexyl)-2-(7-hydroxy-2-oxo(2H-chromen-4-yl))acetamide (32a).

(7-Hydroxy-2-oxo-2H-chromen-4-yl)-acetic acid methyl ester (**1**) was synthesized according to Baker et al. (*J. Chem. Soc.*; 1950; 170, 173.).

A solution of **31** (2.0 g, 8.5 mmol) and 6-aminohexanol (4.0 g, 34.1 mmol) in 15 mL of DMF was heated at 80°C for 24 h. DMF was evaporated under vacuum to afford the mixture of the product and the excess 6-aminohexanol as a viscous syrup. Chromatography on silica eluting with 10% MeOH/CH_2Cl_2 and evaporation of the pure product fractions afforded a white solid which was washed with ether and dried under vacuum. The yield was 2.05 g (75%).

4-{[N-(6-Hydroxyhexyl)carbamoyl]methyl}-2-oxo-2H-chromen-7-yl 2,2-dimethylpropanoate (33a).

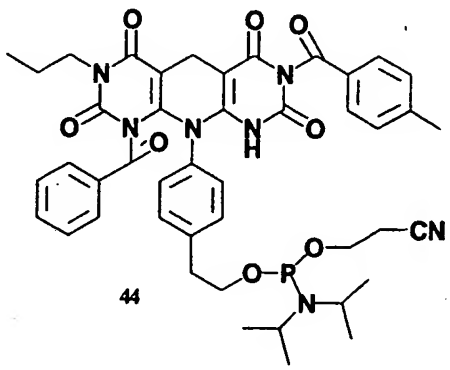
To a solution of **32a** (2.0 g, 6.3 mmol) in 20 mL of dry pyridine was added 4,4'-dimethoxytriphenylmethyl chloride (3.0 g, 8.9 mmol). The solution was kept at room temperature for 1 h, TLC analysis (ethyl acetate, R_f~ 0.7) showed complete reaction (protection of the primary hydroxy group).

(decomp). ^{1}H NMR (DMSO-d_6) δ 0.88 (t, 3H, J = 7.2 Hz, CH_3), 1.27 (m, 2H, CH_2), 1.48 (m, 2H, CH_2), 2.81-2.90 (m, 2H, CH_2), 3.71-3.85 (m, 4H, CH_2), 4.50 (br. s, 5H, OH, NH, H_2O), 7.26 (d, 2H, J = 8.4 Hz, ArH), 7.44 (d, 2H, J = 8.4 Hz, ArH), 8.62 (s, 1H, 5-H).

3-n-Butyl-5,10-dihydro-10-[(2-hydroxyethyl)phenyl]pyrido[2,3-d;6,5-d']dipyrimidine-2,4,6,8-(1H,3H,7H,9H,10H)-tetrone. 42

To a suspension **41** (7.91 g, 18.7 mmol) in 300 ml of 25% aq. NH_3 was added $Na_2S_2O_4$ (13.8 g, 85%, 67 mmol) and slowly heated to 60° with stirring. The mixture was stirred at 60° for 40 min, diluted with water (100 ml) and stirred for additional 1 hr at the same temperature. A clear solution formed. The solution was partially evaporated to one half of its original volume, cooled with ice and neutralized with 50 ml of acetic acid to pH 5 to form a precipitate. The mixture was kept in refrigerator for complete crystallization, filtered and washed with cold water. The solid was dried in vacuum to yield 7.32 g (92%) of **42** as a white solid, mp 182-210 °C (decomp). ^{1}H NMR (DMSO-d_6) δ 0.86 (t, 3H, J = 7.3 Hz, CH_3), 1.23 (m, 2H, CH_2), 1.42 (m, 2H, CH_2), 2.80 (t, 2H, J = 6.6 Hz, CH_2), 3.14 (s, 2H, 5-CH_2), 3.68 (m, 4H, $ArCH_2CH_2$), 4.64 (t, 1H, OH), 7.25 (d, 2H, J = 8.3 Hz, ArH), 7.33 (d, 2H, J = 8.3 Hz, ArH), 7.73 (br. s, 3H, NH).

***PPT* 44**



44

Solid **42** (1.2 g, 2.82 mmol) was evaporated with pyridine (10 ml), suspended in pyridine (13 ml), treated with Me_3SiCl (2.2 ml, 17.3 mmol) and

stirred under argon at ambient temperature for 30 min. The reaction mixture was cooled with ice and treated slowly with toluoyl chloride (5 ml, 28.8 mmol). Stirring was continued at room temperature for 2 hr, and the solvent evaporated. The residue was treated with acetic acid (10 ml) followed by addition of water (10 ml). Precipitated oil was extracted with hexanes (3x50 ml), and the residue that was insoluble in hexanes was evaporated with water. The residue was suspended in 96% ethanol (10 ml) and filtered to recover 0.3 g of the starting material. The mother liquor was diluted with water to precipitate bis-toluoyl derivative as an oil. The oil was dried in vacuum to give 0.96 g (52%) of **43** as a solid foam. This compound without further purification was converted into phosphoramidite by the following procedure. The solid was evaporated with acetonitrile, dissolved in 25 ml of dichloromethane, treated with diisopropylammonium tetrazolide (0.54 g, 3.13 mmol) followed by 2-cyanoethyl tetraisopropylphosphorodiamidite (0.88 g, 2.9 mmol). The reaction mixture was stirred under argon for 1 hr, treated with methanol (1 ml), taken into EtOAc (100 ml), washed with sat. NaCl solution and dried over Na_2SO_4. The solution was evaporated, purified by HPLC on silica gel column using a gradient system 0-50% B; CH_2Cl_2-hexanes-NEt_3 (15:30:1) (A); EtOAc (B); detected at 320 nm. The main fraction was evaporated giving a colorless foam, 0.79 g (33%) of AG1 phosphoramidite **44**. ^1H NMR ($CDCl_3$) δ 0.92 (t, 3H, J = 7.3 Hz, CH_3), 1.07-1.42 (m, 14H, 4xCH_3 (i-Pr), CH_2 (Bu)), 1.50-1.65 (m, 2H, CH_2 (Bu)), 2.35-2.60 (m, 2H, CH_2CN), 2.40 (s, 3H, CH_3Ar), 2.46 (s, 3H, CH_3Ar), 2.95-3.13 (m, 4H, 2xCH (i-Pr), CH_2 (Bu)), 3.45-3.60 (m, 2H, OCH_2), 3.80-4.02 (m, 4H, $ArCH_2CH_2$), 3.82 (s, 2H, 5-CH_2), 7.15-7.35 (m, 8H, ArH (Tol)), 7.45 (s, 1H, NH), 7.73 (br. s, 3H, NH), 7.95 (d, 2H, J = 8.0 Hz, ArH), 8.05 (d, 2H, J = 8.0 Hz, ArH). ^{31}P NMR ($CDCl_3$) δ (ppm, H_3PO_4) 143.2 (s).

Example 11

N-{3-[4-[(1Z)-1-aza-2-(dimethylamino)prop-1-enyl]-1-(5-{[bis(4-

methoxyphenyl)phenylmethoxy}methyl}-4-{[bis(methylethyl)amino](2-cyanoethyl)phophinooxy}oxolan-2-yl)pyrazolo[5,4-d]pyrimidin-3-yl]propyl}[2-({4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)-ethoxy]carboxamide (50) (Reaction Scheme 11)

4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-3-(3-trifluoroacetimido-propyn-1-yl) pyrazolo[3,4-d]pyrimidine (46; n=1).

To a mixture of **45** (1.96 g, 5.20 mmol), CuI (103 mg, 0.54 mmol) and tetrakis[triphenylphosphine]palladium[0] (317 mg, 0.276 mmol) in 10 ml of anhydrous DMF was added anhydrous triethylamine (1.1 ml) followed by propargyl trifluoroacetimide (1.50g, 9.88 mmol). The reaction mixture was stirred under argon for 4 h. The solvent DMF was removed by evaporation and the residual oil was purified by silica gel chromatography eluting with 7% methanol in ethyl acetate. The product fractions were pooled and evaporated affording a foam: 2.16 g (99%) yield.

4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-3-(3-aminopropyl)pyrazolo[3,4-d]pyrimidine (47; n=1).

To a solution of **46** (2.10 g, 5.25 mmol) in 50 ml of ethanol, containing 0.300 mg of 5% palladium on carbon (preactivated with formic acid), was added 1.0 ml of 4 M triethylammonium formate buffer (pH 6.5). The mixture was shaken under 40 psi of hydrogen gas for 18 h. The mixture was filtered through Celite and the filtrate was evaporated affording a solid. 1.8 g (85%) yield.

The solid was stirred in 15 ml of concentrated ammonium hydroxide (sealed flask) for 12 h and then evaporated to dryness. The solid (**47**) was evaporated from dry acetonitrile and stored under vacuum: 1.74 g yield.

Synthesis of 48 (n=1, q=2, R_5=CH_3CH_2-, R_5, R_5=H, R_1=2-Cl, R_5=4-NO_2).

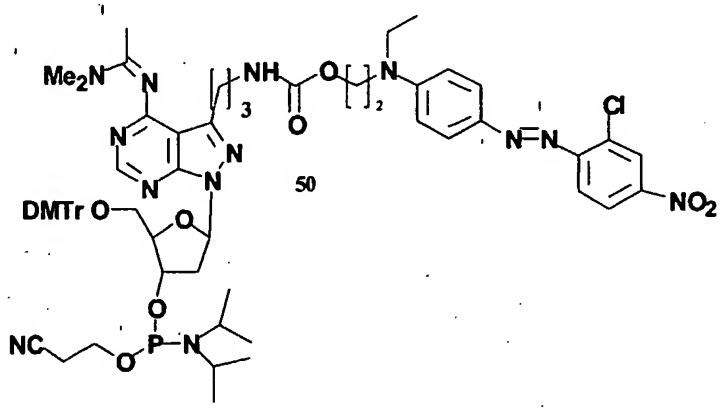
A solution of **47** (0.90 g, 2.92 mmol) and 7 (1.59 g, 2.92 mmol) was

stirred in 5.0 ml of anhydrous dimethylformamide, containing 1.0 ml of triethylamine, at 50 °C for 1.0 h. The solution was evaporated to dryness and the residue was purified by silica gel chromatography eluting with a gradient of 0-20% methanol in ethyl acetate. The product fractions were evaporated affording an amorphous solid: 0.74 g (37%) yield.

Synthesis of 49 (n=1, q=2, R_5=CH_3CH_2-, R_5, R_5=H, R_1=2-Cl, R_5=4-NO_2).

To a solution of **48** (0.71 g, 1.03 mmol) and N,N-dimethylacetamide dimethylacetal (1.9 ml) in 5.0 ml of dimethylacetamide was added 2.0 ml of triethylamine. The solution was stirred for 18 hrs and then evaporated to dryness affording an oil: 0.75 g (100%) yield.

Synthesis of 50 (n=1, q=2, R_5=CH_3CH_2-, R_5, R_5=H, R_1=2-Cl, R_5=4-NO_2).



Dimethoxytrityl chloride (0.42 g) was added to a solution of **49** (0.75 g, 1.03 mmol) in 10 ml of dry pyridine. The solution was stirred for 4.0 hr under argon and then poured into 200 ml of 5% sodium bicarbonate solution. The product was extracted with 300 ml of ethyl acetate. The extract was dried over sodium sulfate and evaporated. The residue was purified by silica gel chromatography eluting with 10% methanol in ethyl acetate (1% triethylamine). The product fractions were evaporated affording a foam: 556 mg (57%) yield.

To a solution of the 5'-dimethoxytrityl derivative (540 mg, 0.567 mmol) in 15 ml of anhydrous methylene chloride, containing 0.30 ml of

diisopropylethylamine, was added 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.25 ml). After stirring for 30 minutes under argon at 25 °C the solution was treated with 1.0 ml of methanol and diluted with 200 ml of ethyl acetate. The solution was washed with 200 ml of 5% sodium bicarbonate solution and dried over sodium sulfate and evaporated. The crude product was purified by silica gel chromatography eluting with 5% methanol in ethyl acetate (2% triethylamine). The product fractions were evaporated affording a foam: 453-mg (76%) yield.

Example 12

Synthesis of fluorogenic oligodeoxynucleotide probes.

The 3'- DPI_3 probes were prepared by automated DNA synthesis from a DPI_3-modified glass support using methods described earlier (Lukhtanov et al. *Biorg. Chem.*, **7:** 564-567 (1996)). Oligonucleotide synthesis was performed on an ABI 394 synthesizer according to the protocol supplied by the manufacturer except that 0.015 M (instead of the standard 0.1 M) iodine solution was utilized in the oxidation step to avoid iodination of the $CDPI_3$ moiety. To prevent extension during PCR, probes without 3'- $CDPI_3$ were prepared with the 3'-hydroxyhexyl phosphate as previously described (Gamper et al. *Biochem.* **36:** 14816-14826 (1997)). The quencher phosphoramidites were added to the CPG and standard β-cyanoethylphosphoramidites and reagents (Glen Research, Sterling, VA) were used in oligonucleotide synthesis. 6-Carboxyfluorescein (6-FAM) phosphoramidite (Glen Research) was used to introduce the 5'-reporter dyes. Alternatively TAMRA-dU phosphoramidite (Glen Research), cy3 or cy5 phosphoramidite (Glen Research), resorufin phosphoramidite, coumarin phosphoramidite, or AG phosphoramidite was used to introduce the indicated 5'-fluorophore. 5'-Hexylamine phosphoramidite (Glen Research) was incorporated into certain ODNs for post-synthetic conjugation of the 3'-quencher dye tetramethylrhodamine (TAMRA). After deprotection, all

oligonucleotides were reverse-phase HPLC purified and isolated as the sodium salts by butanol concentration / sodium perchlorate precipitation (Milesi et al. *Methods Enzym.* **313:** 164-173 (1999)).

Example 13

Post-synthetic conjugation of ODNs with TAMRA.

TAMRA NHS ester (Glen Research) was used to acylate the hexylamine linkers in certain ODNs according to the protocol supplied by the manufacturer. The resulting $CDPI_3$-probes with two conjugated dyes were purified by denaturing gel electrophoresis using 8% polyacrylamide. The desired bands were excised and the gel slices were incubated overnight at 37°C in 10 mL of 100 mM Tris-HCl, 10mM triethylammonium chloride, 1 mM EDTA (pH 7.8). The products were isolated from the extract by reverse phase HPLC, butanol concentration and sodium perchlorate precipitation. The pellets were dissolved in water and the concentrations were determined spectrophotometrically. A nearest neighbor model (Cantor, et al. Biopolymers **9:** 1059 - 1077 (1970) was applied to calculate extinction coefficients (ε_{260}) of ODNs. For the conjugates and probes, extinction coefficients were calculated as a sum of ε_{260} for the ODN and the incorporated residues of DPI_3 (68,000 M^{-1}, cm^{-1}), 6-FAM (22,800 M^{-1}, cm^{-1}), TAMRA (34,000 M^{-1}, cm^{-1}) and quencher (11,300 M^{-1}, cm^{-1}).

Example 14

Digestion of oligonucleotides by Snake Venom Phosphodiestaerase.

Oligonucleotides were digested with snake venom phosphodiesterase (PDE) to study the fluorescence quenching potential of various quenchers. 200 nM of oligonucleotide was taken in a buffer containing 40 mM of NaCl, 20 mM of Tris (pH 8.9) , 5mM of $MgCl_2$ and 0.025% of BSA. Initial fluorescence was read on a LS50B fluorimeter (Perkin-Elmer Corporation, Foster City, CA) before the addition of phosphodiesterase (Pharmacia,

Piscataway, NJ) 54 units of enzyme was added to the reaction mixture and incubated at 37 ^{0}C for 16 hrs. The final fluorescence was then measured using the LS50B. The ratio of final fluorescence to the initial fluorescence represents the signal to noise ratio (S/N) of the quenchers. Independently the kinetics of digestion reactions were monitored using the LS50B to determine the time required for complete digestion of oligonucleotides.

Example 15

5' Nuclease PCR Assay.

$CDPI_3$-conjugated oligonucleotides were conjugated with a fluorophore, FAM at the 5' end and various quenchers were conjugated through a linker at the 3' end by the methods discussed above. 5' nuclease assays were performed with the above oligonucleotides to determine the quenching ability of the various quenchers under investigation. Fluorescent monitoring was performed in an Idaho Technologies LC-24 LightCycler. Each reaction contained PCR buffer (40 mM NaCl, 20 mM Tris HCl, pH 8.9, 5 mM $MgSO_4$, 0.05% bovine serum albumin), 125 mM each dNTP, 0.5 mM each primer, 0.1 mM fluorescent $CDPI_3$ probe, 0.5 U/10 mL *Taq* polymerase and 0.1 ng/10 mL of synthetic DNA as template. The cycling program was 50 cycles (or as indicated) of 2 sec at 95EC, then 30 sec at the extension temperature (55-70E).

WHAT IS CLAIMED IS:

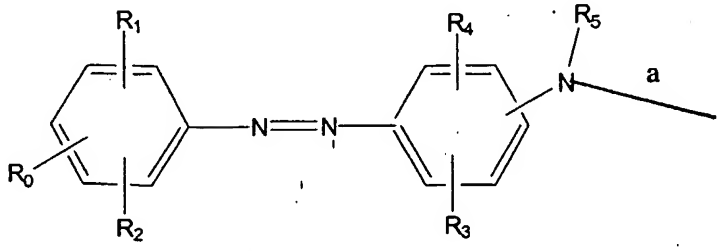
1. An oligonucleotide conjugate having the formula

FL-ODN-Q

where ODN is an oligonucleotide or nucleic acid;

FL is a fluorophore moiety covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, and

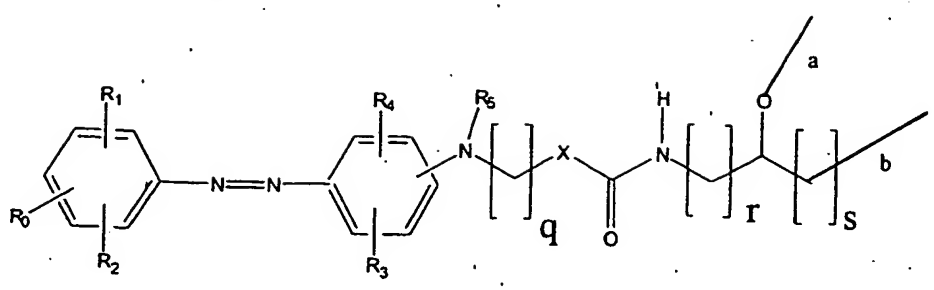
Q is a quencher moiety covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, the quencher moiety having the structure



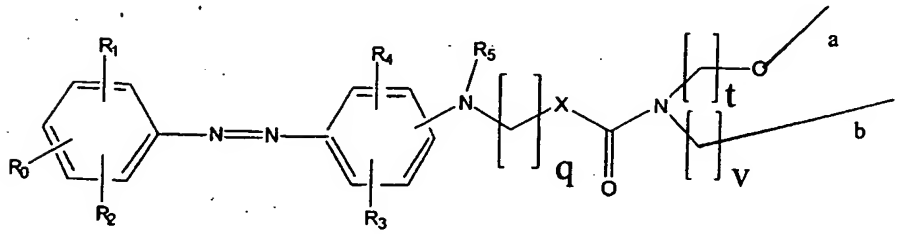
where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$ where n= 0 to 5, $-NO_2$, $-SO_3$, $-N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or $-(CH_2)_{n''}CH_3$ where n''= 0 to 5, and where the quencher moiety is attached to the linker through the valence bond designated a.

2. An oligonucleotide conjugate in accordance with Claim 1 where R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is H or Cl in the 2 position of the benzene nucleus, and R_3 and R_4 are hydrogen and R_5 is ethyl.

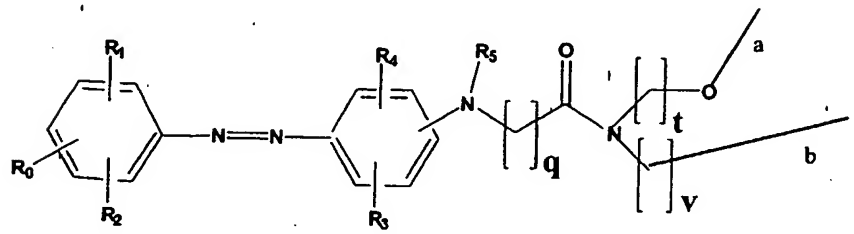
3. An oligonucleotide conjugate in accordance with Claim 1 where the quencher moiety and the linker attaching it to the ODN comprises the structures selected from the moieties shown by the formulas Q-1, Q-2 and Q-3



Q-1



Q-2

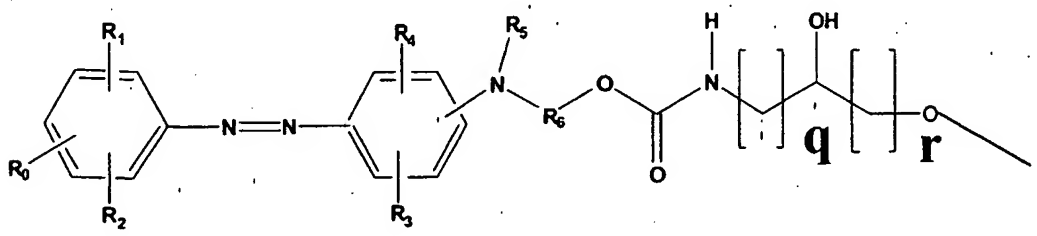


Q-3

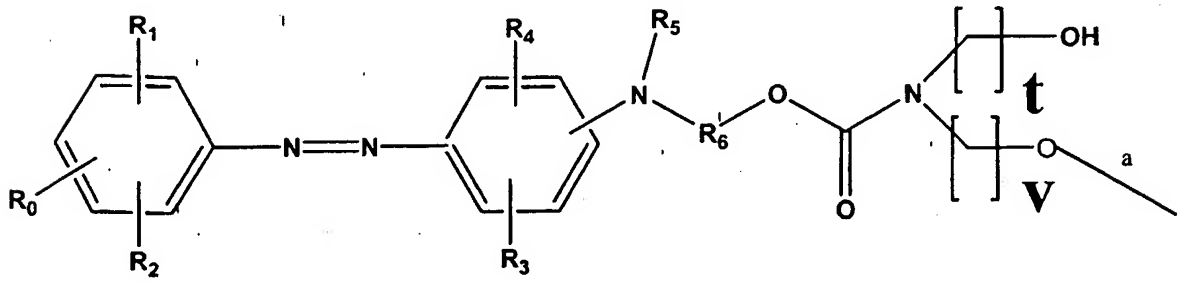
where q is 1 to 20, X is –O-, -OCH_2- or -CH_2-; t and v independently are 1 to 20, r and s independently are 1 to 20, and the conjugated quencher and linker moiety is attached to the ODN through one of the valence bonds designated a or b.

4. An oligonucleotide conjugate in accordance with Claim 3 further comprising a minor groove binder moiety attached to the quencher-linker conjugate through one of the valence bonds designated a or b.

5. An oligonucleotide conjugate in accordance with Claim 1 where the quencher moiety and of the linker attaching it to the ODN comprises the structures selected from the moieties shown by the formulas Q-4, and Q-5



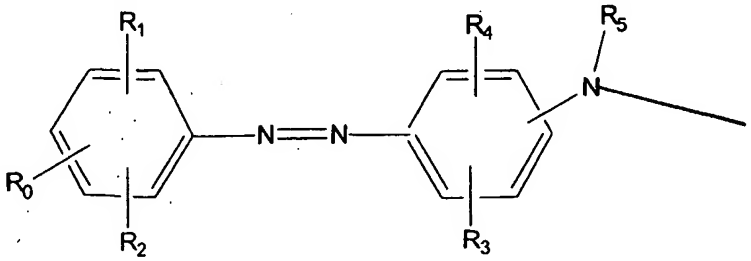
Q-4



Q-5

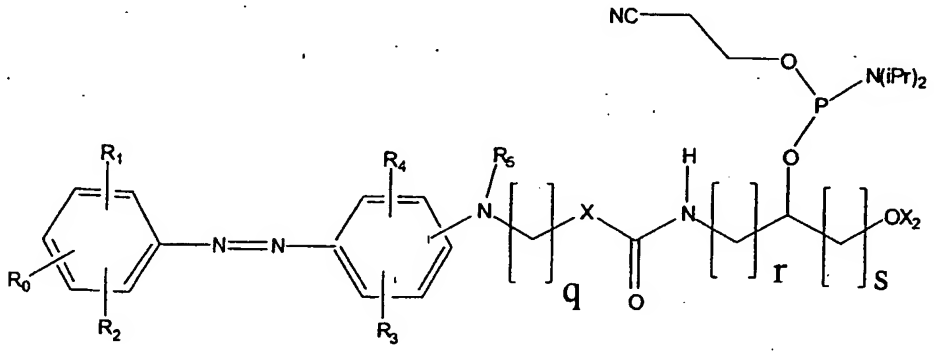
where R_6 is $-(CH_2)_{n*}$ where n* is 1 to 20, and t and v independently are 1 to 20, and where the quencher moiety is attached to the ODN through the valence bond designated a.

6. A phosphoramidite reagent for preparing an oligonucleotide-fluorophore-quencher conjugate, the reagent including the moiety

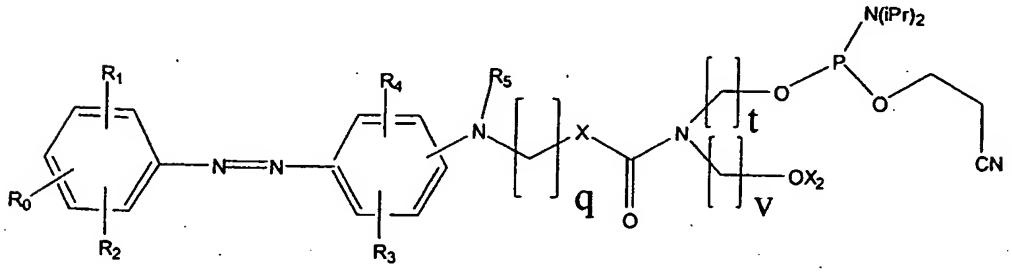


where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_nCH_3$, -$(CH_2)_nCH_3$ where n= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or -$(CH_2)_{n''}CH_3$ where n"= 0 to 5, and a bis(methylethyl)amino](2-cyanoethoxy)phosphinooxy moiety covalently linked thereto.

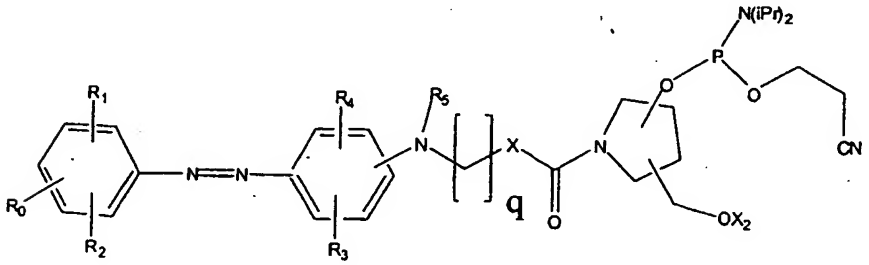
7. A phosphoramidite reagent in accordance with Claim 6 having the formula selected from the group consisting of the formulas designated PA-1, PA-2 and PA-3



PA-1



PA-2



PA-3

where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen, -$O(CH_2)_nCH_3$, -$(CH_2)_nCH_3$ where n= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or -$(CH_2)_{n''}CH_3$ where n"= 0 to 5, q is 1

to 20, X is –O- or -CH_2-; t, v, r and s independently are 1 to 20, and X_2 is H or dimethoxytrityl, methoxytrityl, trityl or an acid labile blocking group.

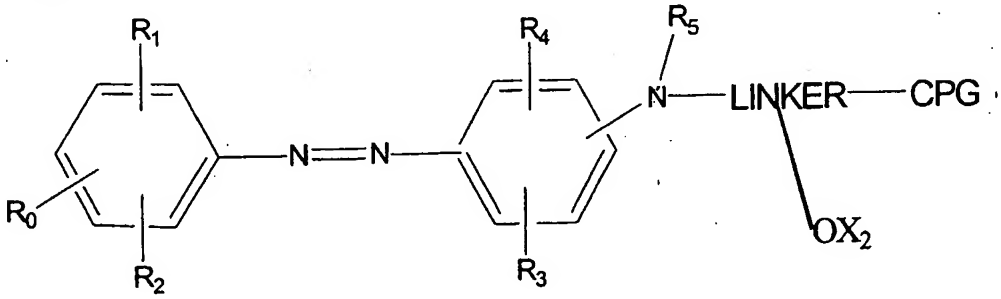
8. A phosphoramidite reagent in accordance with Claim 7 that has the formula designated PA-1.

9. A phosphoramidite reagent in accordance with Claim 7 that has the formula designated PA-2.

10. A phosphoramidite reagent in accordance with Claim 7 that has the formula designated PA-3.

11. A phosphoramidite reagent in accordance with Claim 7 where R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is Cl in the 2 position of the benzene nucleus, and R_3 and R_4 are hydrogen and R_5 is ethyl.

12. A covalently linked solid support and quencher conjugate suitable for oligonucleotide synthesis, having the structure



where CPG stands for a polymeric solid support;

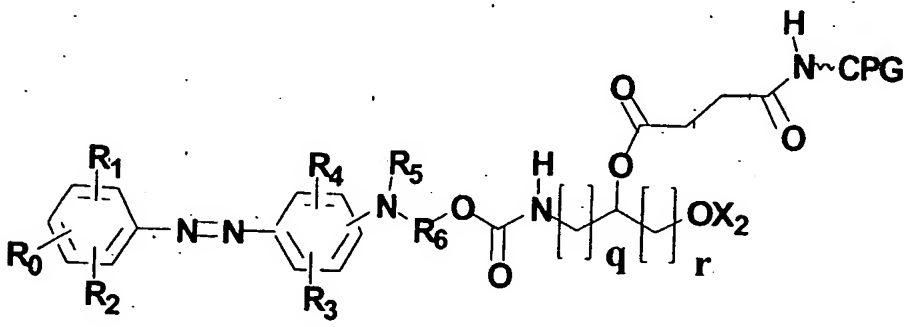
LINKER is a moiety having the length of 1 to approximately 30 atoms and linking the diphenylazo moiety to the CPG;

X_2 is OH or , dimethoxytityl, methoxytrityl, trityl or an acid labile blocking group;

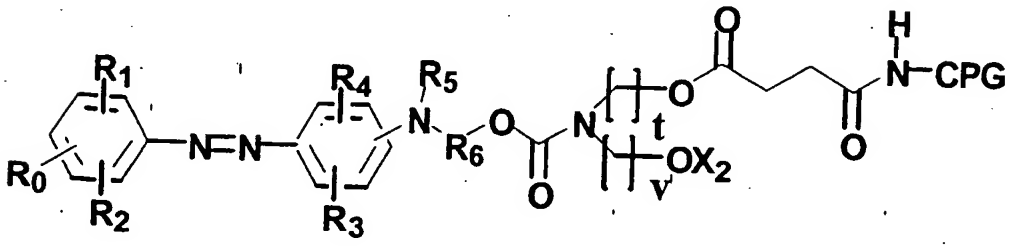
R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_nCH_3$,

$-(CH_2)_nCH_3$ where n= 0 to 5, $-NO_2$, $-SO_3$, $-N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or $-(CH_2)_{n''}CH_3$ where n"= 0 to 5.

13. A covalently linked solid support and quencher conjugate in accordance with Claim 12 selected from the structures



and



where R_6 is $-(CH_2)_{n*}$ where n* is 1 to 20, and q, r, t and v independently are 1 to 20.

14. A covalently linked solid support and quencher conjugate in accordance with Claim 13 where R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is Cl in the 2 position of the benzene nucleus, and R_5 is ethyl.

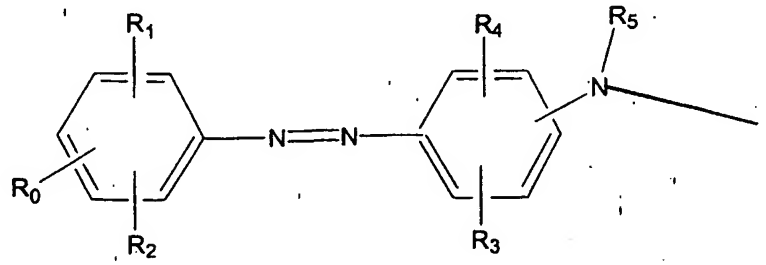
15. An oligonucleotide conjugate having the formula

FL-ODN-Q- MGB

where ODN is an oligonucleotide or nucleic acid;

FL is a fluorophore covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, and

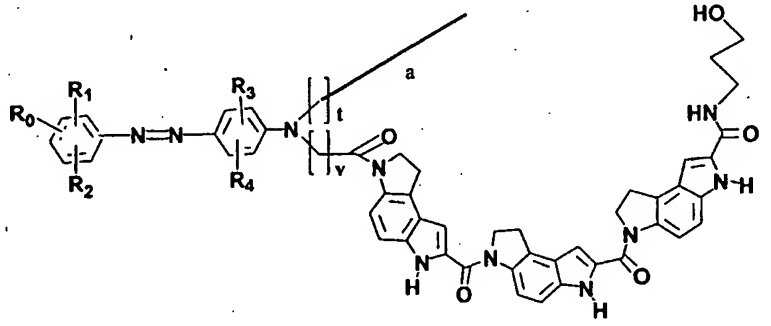
Q is a quencher moiety covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, the quencher moiety having the structure



where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_nCH_3$, -$(CH_2)_nCH_3$ where n= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H, -$(CH_2)_{n''}CH_3$ or -$(CH_2)_{n''}$- where n"= 0 to 5, and

MGB is minor groove binder moiety covalently attached to the ODN moiety or to the quencher moiety through a linker having the length of 0 to approximately 30 atoms.

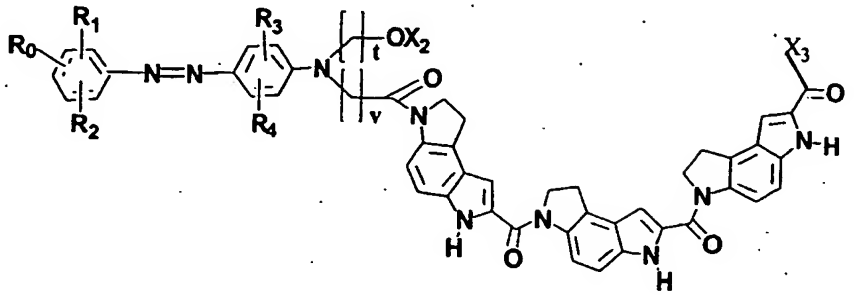
16. An oligonucleotide conjugate in accordance with Claim 15 where the MGB moiety is attached to the quencher moiety, and the covalently bonded MGB -Q moiety has the structure



where t and v independently are 1 to 20, and the valence bond designated **a** attaches the MGB-Q moiety to the ODN moiety.

17. An oligonucleotide conjugate in accordance with Claim 16 where R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is H or Cl in the 2 position of the benzene nucleus, and R_3 and R_4 are hydrogen.

18. A covalently bonded minor groove binder and quencher reagent for oligonucleotide synthesis, having the formula



where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_nCH_3$, -$(CH_2)_nCH_3$ where n= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and t and v independently are 1 to 20;

X_2 is H or dimethoxytrityl, methoxytrityl, trityl or an acid labile blocking group, and

X_3 is pentafluorophenyloxy, or NH-LINKER-CPG or O-LINKER-CPG where CPG is a polymeric solid support and LINKER is a linking moiety having a length of approximately 0 to 30 atoms linking the tricyclic moiety to the CPG.

19. A covalently bonded minor groove binder and quencher reagent in accordance with Claim 18 wherein X_3 is pentafluorophenyloxy.

20. A covalently bonded minor groove binder and quencher reagent in accordance with Claim 18 wherein X_3 is NH-LINKER-CPG or O-LINKER-CPG.

21. A covalently bonded minor groove binder and quencher reagent in accordance with Claim 18 where R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is H or Cl in the 2 position of the benzene nucleus, R_3 and R_4 are hydrogen and v=t=3.

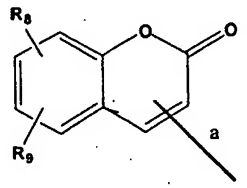
22. An oligonucleotide conjugate having the formula

FL-ODN-Q

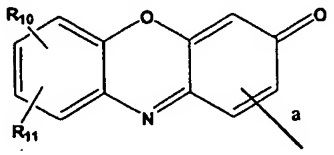
where ODN is an oligonucleotide or nucleic acid;

Q is a quencher moiety covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, and

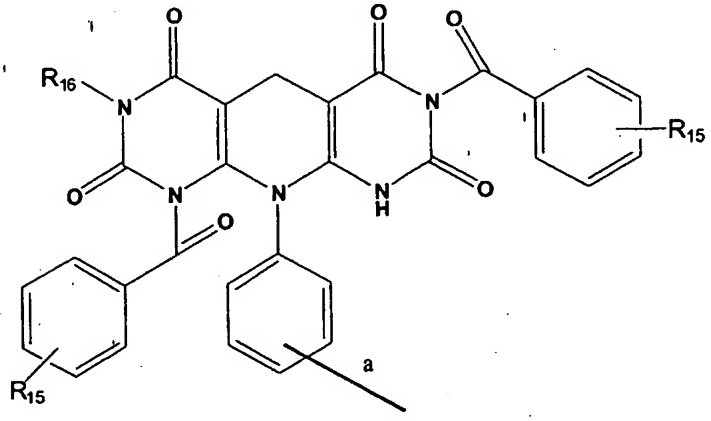
FL is a fluorophore covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, said fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

R_{10} and R_{11} independently are H, $-CN$, $-OR_{12}$, $-N(R_{12})_2$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated a symbolizes covalent attachment of the fluorophore to the linker.

23. An oligonucleotide conjugate in accordance with Claim 22 where the fluorophore has the formula designated FL-1.

24. An oligonucleotide conjugate in accordance with Claim 23 where R_8 is $OC(O)CH(CH_3)_2$ and R_9 is H.

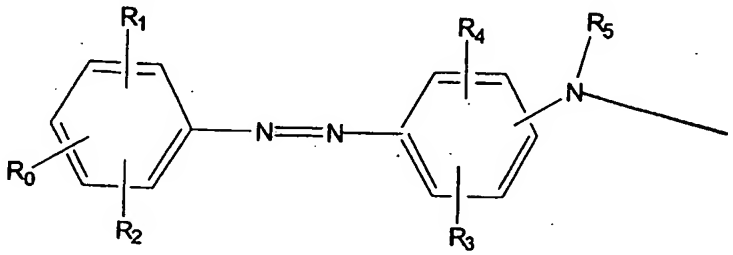
25. An oligonucleotide conjugate in accordance with Claim 22 where the fluorophore has the formula designated FL-2.

26. An oligonucleotide conjugate in accordance with Claim 25 where R_{10} is $OC(O)CH(CH_3)_2$ and R_{11} is H.

27. An oligonucleotide conjugate in accordance with Claim 22 where the fluorophore has the formula designated FL-3.

28. An oligonucleotide conjugate in accordance with Claim 28 where R_{15} is methyl and R_{16} is *n*-propyl.

29. An oligonucleotide conjugate in accordance with Claim 22 where the quencher moiety comprises the structure

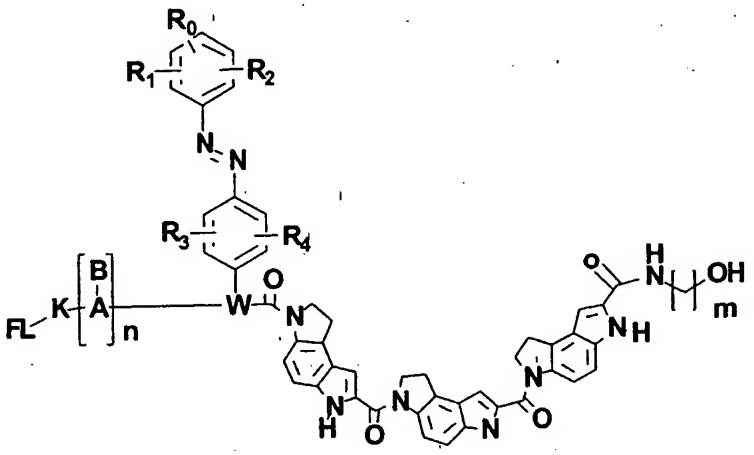


where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,$-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$ where n= 0 to 5, $-NO_2$, $-SO_3$, $-N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or $-(CH_2)_{n''}CH_3$ where n"= 0 to 5.

30. An oligonucleotide conjugate in accordance with Claim 22 comprising an additional minor groove binder moiety (MGB) attached to the quencher moiety through a linker having the length of 0 to approximately 30 atoms, whereby the oligonucleotide conjugate has the formula

FL-ODN-Q-MGB.

31. An oligonucleotide conjugate of the formula



wherein R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_{n*}CH_3$, -$(CH_2)_{n*}CH_3$ where n*= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN;

FL is a fluorophore moiety with emission wavelengths in the range of about 300 to about 800 nm;

K is a linker containing 1 to approximately 30 atoms selected from the group consisting of C, O, N, S, P and H;

$[A-B]_n$ symbolizes an ODN, DNA, RNA or PNA or any combination thereof, where **A** is the sugar phosphate backbone where the sugar and the phosphate may independently be modified; **B** is a heterocyclic base, where **B** is independently selected from purine, pyrimidine, pyrazolo[3,4-d]pyrimidine, 7-substituted pyrazolo[3,4-d]pyrimidine- , 7-deazapurine, 7-substiuted 7-

deazapurine, and modified purine- and pyrimidine-bases, and where the DNA, RNA, PNA or ODN can include any combinations of these bases, and

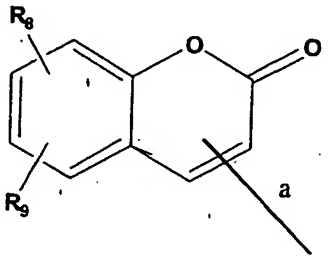
and **n** is the number of nucleotide units in said DNA, RNA, PNA or ODN;

W is a linker of a length of 0 to approximately 30 atoms, selected from the group consisting of C, O, N, S, P and H, and

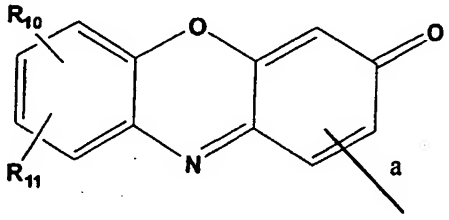
m is an integer having the values of 1 to 20.

32. An oligonucleotide conjugate in accordance with Claim 31 where R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is H or Cl in the 2 position of the benzene nucleus, and R_3 and R_4 are hydrogen.

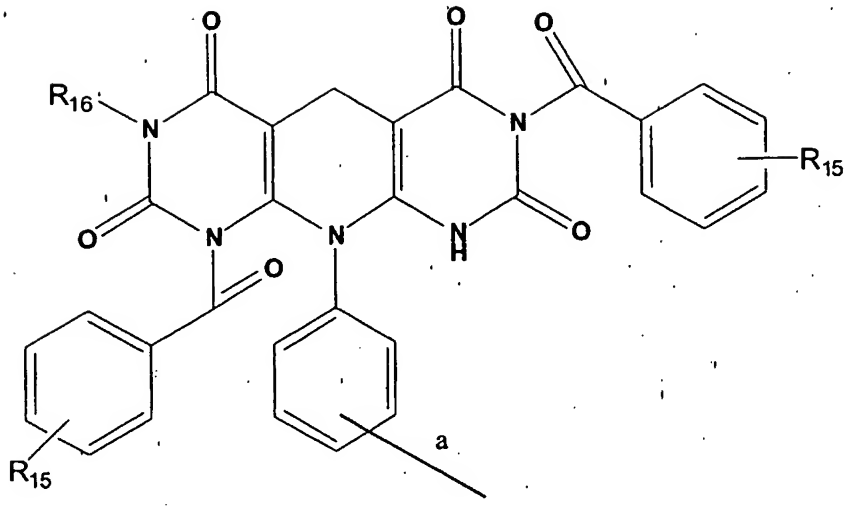
33. An oligonucleotide conjugate in accordance with Claim 31 where said fluorophore moiety has the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

where R_8 is OH or O-alkanoyl where the alkanoyl group has 1 to 10 carbons;

R_9 is H or alkyl of 1 to 10 carbons;

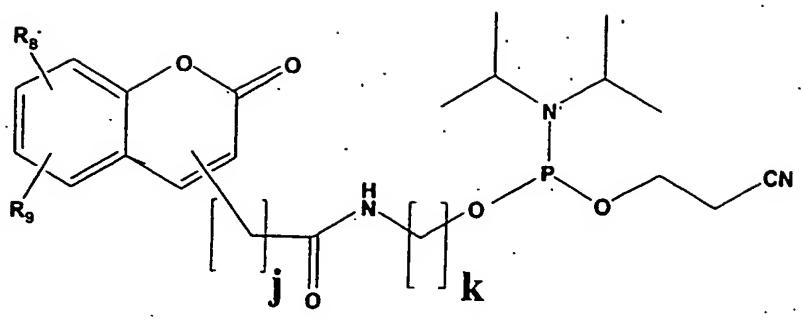
R_{10} and R_{11} independently are H, $-OR_{12}$, $-NHR_{13}$, halogen,$-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ or –CN where n= 0 to 5;

R_{15} is H or alkyl of 1 to 10 carbons;

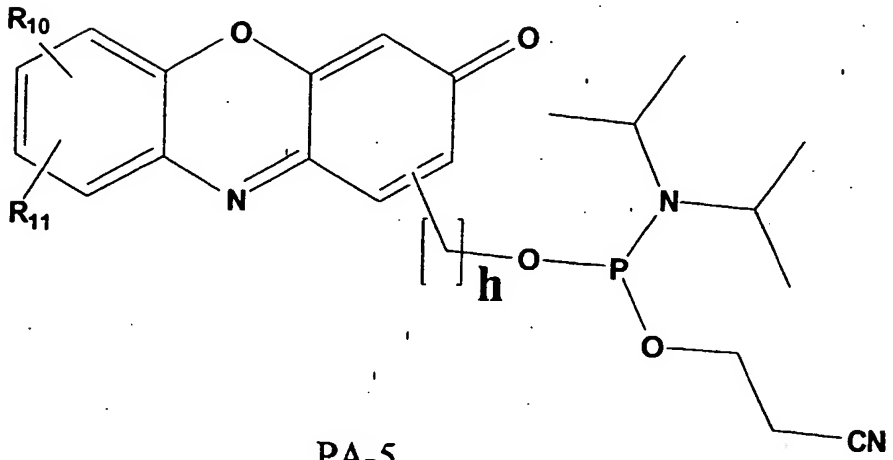
R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated **a** symbolizes covalent attachment of the fluorophore to the linker K.

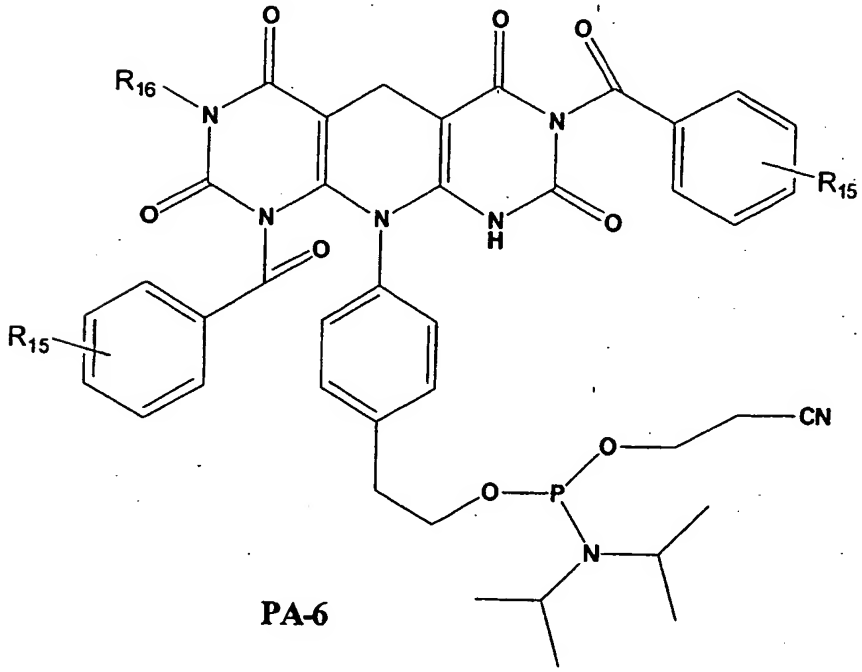
34. A phosphoramidite reagent for preparing an oligonucleotide-fluorophore-quencher conjugate, the reagent selected from the group consisting of the structures designated PA-4, PA-5 and PA-6,



PA-4



PA-5



PA-6

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, a blocking group compatible with oligomer synthesis

removable under acid or alkaline conditions; or an alkyl or alkanoyl group having 1 to 10 carbon atoms;

j and k independently are 1 to 10;

R_{10} and R_{11} independently are H, $-OR_{12}$, $-NHR_{13}$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$, O-alkyl or O-alkanoyl where the alkanoyl group has 1 to 10 carbons, or –CN where n= 0 to 5; h=1 to 20; and R_{12} and R_{13} are blocking groups compatible with ODN synthesis;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons.

35. A phosphoramidite reagent in accordance with Claim 34 that has the formula designated PA-4.

36. A phosphoramidite reagent in accordance with Claim 35 where R_8 is $-OC(O)CH(CH_3)_2$, R_9 is H, j is 2 and k is 6.

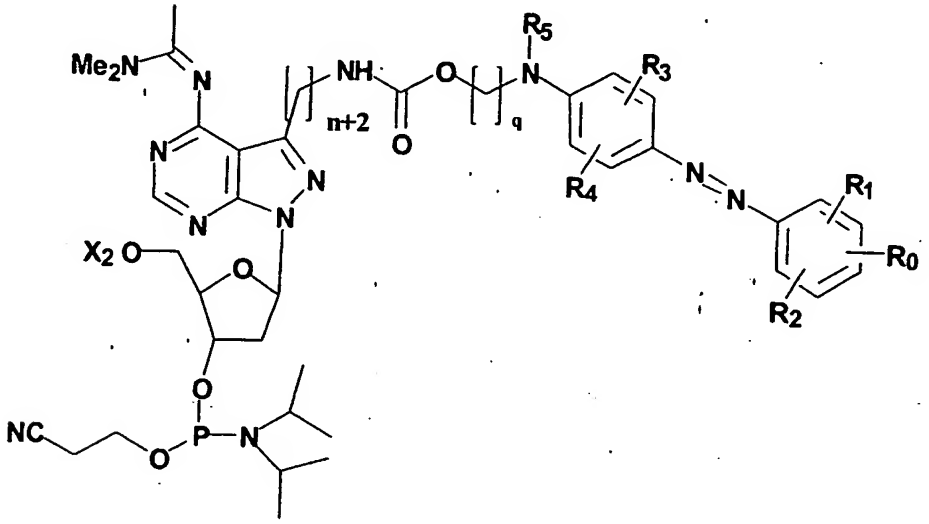
37. A phosphoramidite reagent in accordance with Claim 34 that has the formula designated PA-5.

38. A phosphoramidite reagent in accordance with Claim 37 where R_{10} is $OC(O)CH(CH_3)_2$, R_{11} is H and h is 3.

39. A phosphoramidite reagent in accordance with Claim 34 that has the formula designated PA-6.

40. A phosphoramidite reagent in accordance with Claim 39 where R_{15} is methyl and R_{16} is *n*-propyl.

41. A phosphoramidite reagent for preparing an oligonucleotide-fluorophore-quencher conjugate, the reagent having the formula



wherein R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_{n*}CH_3$, -$(CH_2)_{n*}CH_3$ where n*= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or -$(CH_2)_{n''}CH_3$ where n"= 0 to 5;

n is 1 to 10;

q is 1 to 20, and

X_2 is H or dimethoxytrityl, , methoxytrityl, trityl or an acid labile blocking group.

42. A phosphoramidite reagent in accordance with Claim 41 where R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is Cl in the 2 position of the benzene nucleus, and R_3 and R_4 are hydrogen, R_5 is ethyl, n is 1 and q is 2.

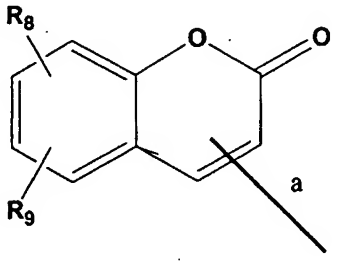
43. An oligonucleotide conjugate having the formula

FL-ODN-MGB

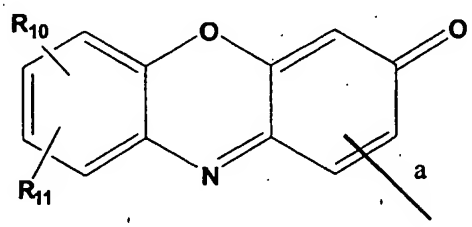
where ODN is an oligonucleotide or nucleic acid;

MGB is minor groove binder moiety covalently attached to the ODN moiety or to the quencher moiety through a linker having the length of 0 to approximately 30 atoms;

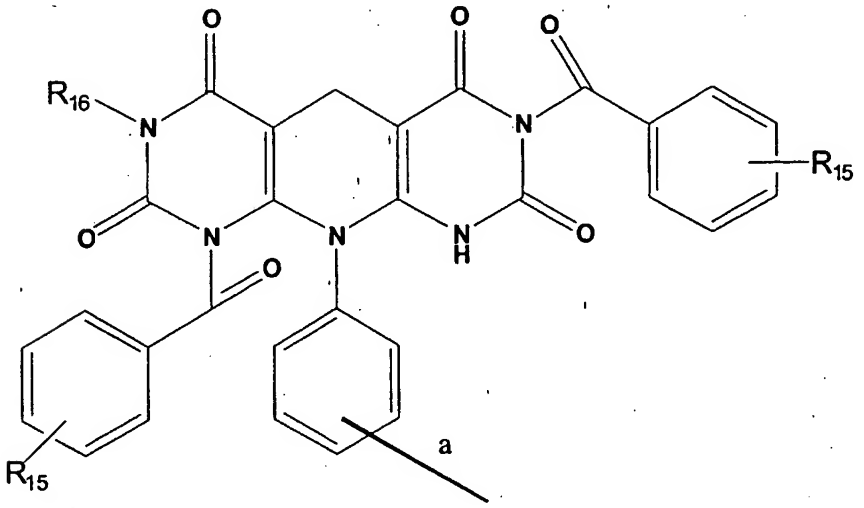
FL is a fluorophore covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, said fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

R_{10} and R_{11} independently are H, $-CN$, $-OR_{12}$, $-N(R_{12})_2$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated a symbolizes covalent attachment of the fluorophore to the linker.

44. An oligonucleotide conjugate in accordance with Claim 43 where the fluorophore has the formula designated FL-1.

45. An oligonucleotide conjugate in accordance with Claim 44 where R_8 is $-OC(O)CH(CH_3)_2$ and R_9 is H.

46. An oligonucleotide conjugate in accordance with Claim 43 where the fluorophore has the formula designated FL-2.

47. An oligonucleotide conjugate in accordance with Claim 46 where R_{10} is $OC(O)CH(CH_3)_2$ and R_{11} is H.

48. An oligonucleotide conjugate in accordance with Claim 43 where the fluorophore has the formula designated FL-3.

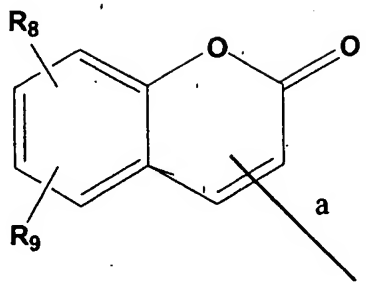
49. An oligonucleotide conjugate in accordance with Claim 49where R_{15} is methyl and R_{16} is *n*-propyl.

50. An oligonucleotide conjugate having the formula

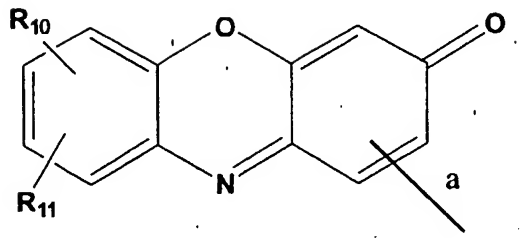
FL-ODN

where ODN is an oligonucleotide or nucleic acid;

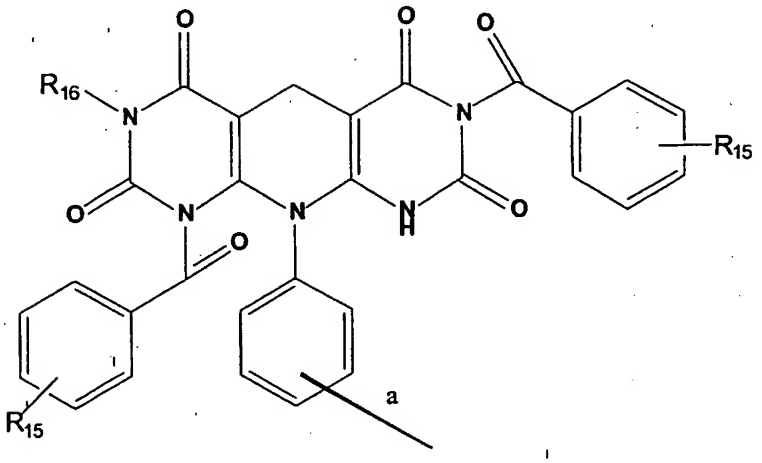
FL is a fluorophore covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, said fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

R_{10} and R_{11} independently are H, $-CN$, $-OR_{12}$, $-N(R_{12})_2$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated **a** symbolizes covalent attachment of the fluorophore to the linker.

51. An oligonucleotide conjugate in accordance with Claim 50 where the fluorophore has the formula designated FL-1.

52. An oligonucleotide conjugate in accordance with Claim 51 where R_8 is $OC(O)CH(CH_3)_2$ and R_9 is H.

53. An oligonucleotide conjugate in accordance with Claim 50 where the fluorophore has the formula designated FL-2.

54. An oligonucleotide conjugate in accordance with Claim 53 where R_{10} is $OC(O)CH(CH_3)_2$ and R_{11} is H.

55. An oligonucleotide conjugate in accordance with Claim 50 where the fluorophore has the formula designated FL-3.

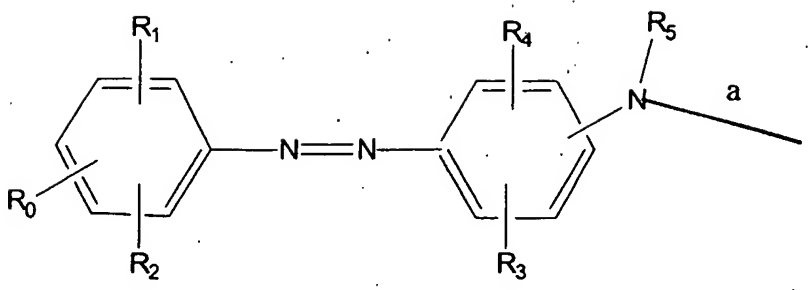
56. An oligonucleotide conjugate in accordance with Claim 55 where R_{15} is methyl and R_{16} is *n*-propyl.

57. A method for hybridizing nucleic acids, comprising the steps of:

(a) providing a first nucleic acid and a second nucleic acid,

(b) incubating the nucleic acids under hybridization conditions, and

(c) identifying hybridized nucleic acids; wherein at least one of the nucleic acids comprises a **FL-nucleic-acid-Q** conjugate where **FL** is a fluorophore moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms,, and **Q** is a quencher moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms, the quencher moiety having the structure



where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_nCH_3$, -$(CH_2)_nCH_3$ where n= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or -$(CH_2)_{n''}CH_3$ where n"= 0 to 5, and where the quencher moiety is attached to the linker through the valence bond designated a.

58. A method in accordance with Claim 57 where in the formula **Q** of the quencher moiety R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is Cl in the 2 position of the benzene nucleus, and R_3 and R_4 are hydrogen and R_5 is ethyl.

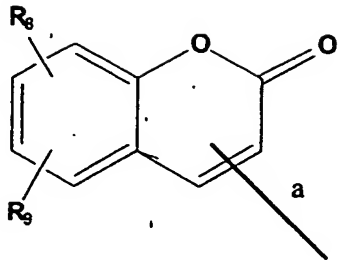
59. A method for hybridizing nucleic acids, comprising the steps of:

(a) providing a first nucleic acid and a second nucleic acid,

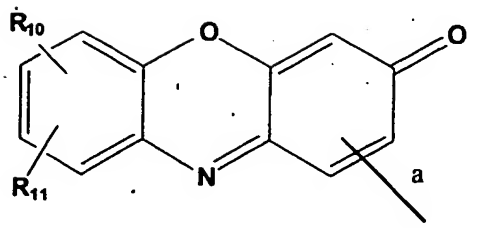
(b) incubating the nucleic acids under hybridization conditions, and

(c) identifying hybridized nucleic acids; wherein at least one of the nucleic acids comprises a **FL-nucleic-acid-Q** conjugate where **Q** is a quencher moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms, and

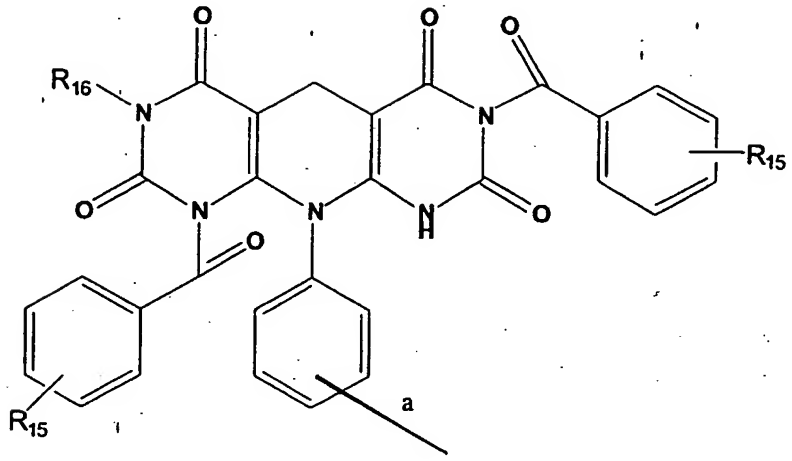
wherein FL is a fluorophore covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, said fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

R_{10} and R_{11} independently are H, $-CN$, $-OR_{12}$, $-N(R_{12})_2$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated **a** symbolizes covalent attachment of the fluorophore to the linker.

60. A method in accordance with Claim 59 where the fluorophore has the formula designated FL-1.

61. A method in accordance with Claim 60 where R_8 is $OC(O)CH(CH_3)_2$ and R_9 is H.

62. A method in accordance with Claim 59 where the fluorophore has the formula designated FL-2.

63. A method in accordance with Claim 62 where R_{10} is $OC(O)CH(CH_3)_2$ and R_{11} is H.

64. A method in accordance with Claim 59 where the fluorophore has the formula designated FL-3.

65. A method in accordance with Claim 64 where R_{15} is methyl and R_{16} is *n*-propyl.

66. A method for discriminating between polynucleotides which differ by a single nucleotide, the method comprising the following steps:

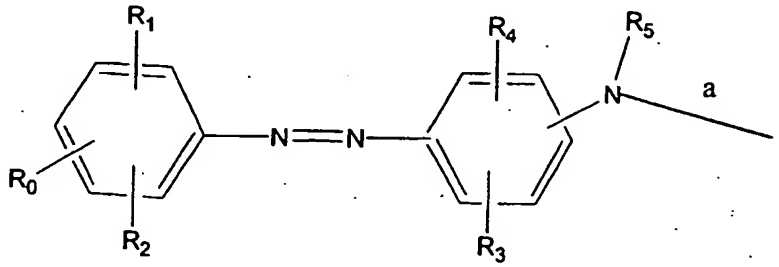
(a) providing a polynucleotide comprising a target sequence,

(b) providing at least two **FL-ODN-Q** conjugates, wherein ODN represents an oligonucleotide moiety, one of the at least two **FL-ODN-Q** conjugates has a sequence that is perfectly complementary to the target sequence and at least one other of the **FL-ODN-Q** conjugates has a single-nucleotide mismatch with the target sequence;

(c) separately incubating each of the **FL-ODN-Q** conjugates with the polynucleotide under hybridization conditions; and

(d) determining the hybridization strength between each of the **FL-ODN-Q** and the polynucleotide, wherein **FL** is a fluorophore moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms,, and **Q** is a quencher moiety covalently attached to

the nucleic acid through a linker having the length of 0 to approximately 30 atoms, the quencher moiety having the structure



where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$ where n= 0 to 5, $-NO_2$, $-SO_3$, $-N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or $-(CH_2)_{n''}CH_3$ where n"= 0 to 5, and where the quencher moiety is attached to the linker through the valence bond designated a.

67. A method in accordance with Claim 66 where in the formula of the quencher moiety **Q** R_o is H, R_1 is NO_2 in the 4 position of the benzene nucleus, R_2 is Cl in the 2 position of the benzene nucleus, and R_3 and R_4 are hydrogen and R_5 is ethyl.

68. A method for discriminating between polynucleotides which differ by a single nucleotide, the method comprising the following steps:

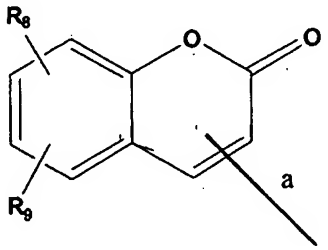
(a) providing a polynucleotide comprising a target sequence,

(b) providing at least two **FL-ODN-Q** conjugates, wherein ODN represents an oligonucleotide moiety, one of the at least two **FL-ODN-Q** conjugates has a sequence that is perfectly complementary to the target sequence and at least one other of the **FL-ODN-Q** conjugates has a single-nucleotide mismatch with the target sequence;

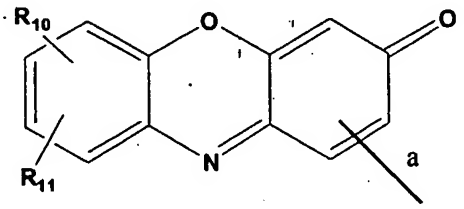
(c) separately incubating each of the **FL-ODN-Q** conjugates with the polynucleotide under hybridization conditions; and

(d) determining the hybridization strength between each of the **FL-ODN-Q** and the polynucleotide, wherein **Q** is a quencher moiety covalently

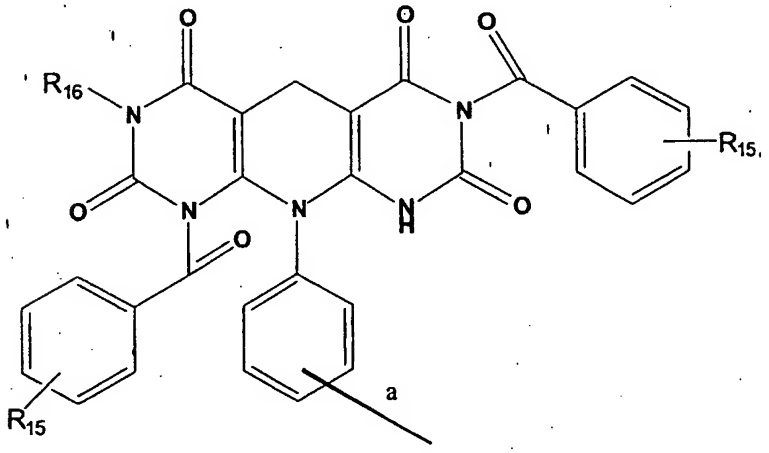
attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms, and **FL** is a fluorophore moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms,, and the fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

R_{10} and R_{11} independently are H, $-CN$, $-OR_{12}$, $-N(R_{12})_2$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated a symbolizes covalent attachment of the fluorophore to the linker.

69. A method in accordance with Claim 68 where the fluorophore has the formula designated FL-1.

70. A method in accordance with Claim 69 where R_8 is $OC(O)CH(CH_3)_2$ and R_9 is H.

71. A method in accordance with Claim 68 where the fluorophore has the formula designated FL-2.

72. A method in accordance with Claim 71 where R_{10} is $OC(O)CH(CH_3)_2$ and R_{11} is H.

73. A method in accordance with Claim 68 where the fluorophore has the formula designated FL-3.

74. A method in accordance with Claim 73 where R_{15} is methyl and R_{16} is *n*-propyl.

75. A method for hybridizing nucleic acids, comprising the steps of:

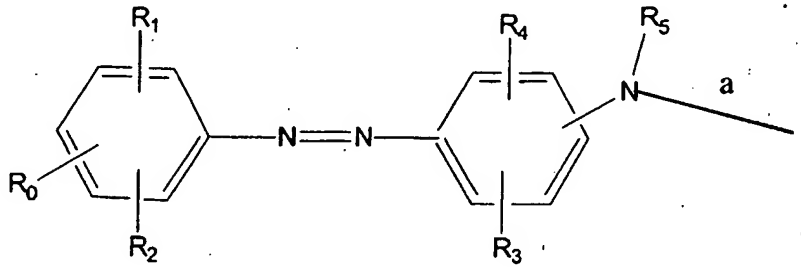
(a) providing a first nucleic acid and a second nucleic acid,

(b) incubating the nucleic acids under hybridization conditions, and

(c) identifying hybridized nucleic acids;

wherein at least one of the nucleic acids comprises a **FL-nucleic-acid-Q-MGB** conjugate where **FL** is a fluorophore moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms, MGB is minor groove binder moiety covalently attached to the ODN

moiety or to the quencher moiety through a linker having the length of 0 to approximately 30 atoms and **Q** is a quencher moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms, the quencher moiety having the structure



where R_0, R_1, R_2, R_3 and R_4 are independently -H, halogen,-$O(CH_2)_nCH_3$, -$(CH_2)_nCH_3$ where n= 0 to 5, -NO_2, -SO_3, -$N[(CH_2)_{n'}CH_3]_2$ where n'= 0 to 5 or –CN, and R_5= -H or -$(CH_2)_{n''}CH_3$ where n"= 0 to 5, and where the quencher moiety is attached to the linker through the valence bond designated a.

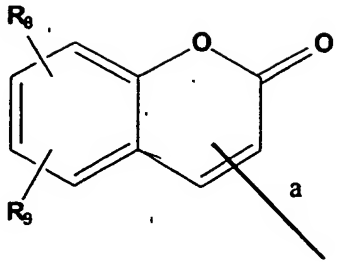
76. A method for hybridizing nucleic acids, comprising the steps of:

(a) providing a first nucleic acid and a second nucleic acid,

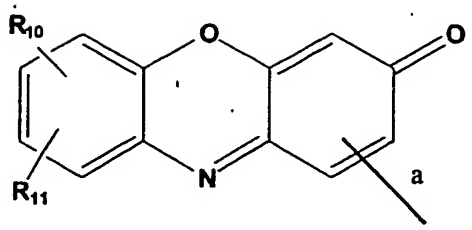
(b) incubating the nucleic acids under hybridization conditions, and

(c) identifying hybridized nucleic acids;

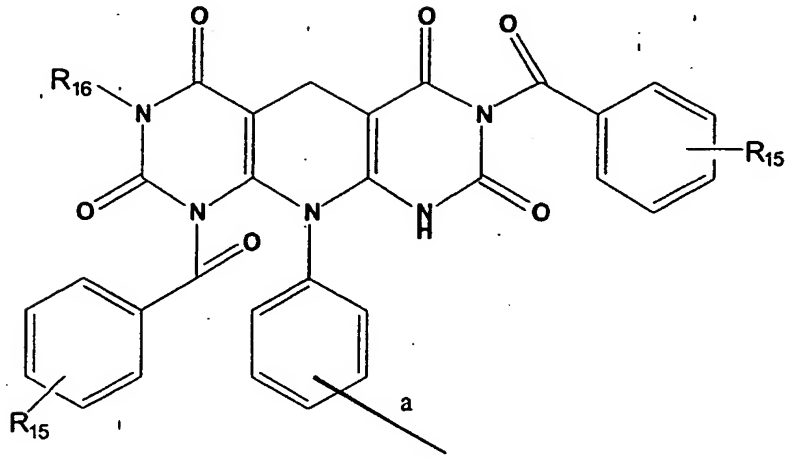
wherein at least one of the nucleic acids comprises a **FL-ODN-Q-MGB** conjugate where ODN is a nucleic acid or modified nucleic acid, MGB is minor groove binder moiety covalently attached to the ODN moiety or to the quencher moiety through a linker having the length of 0 to approximately 30 atoms, **Q** is a quencher moiety covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, and **FL** is a fluorophore moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms, and the fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

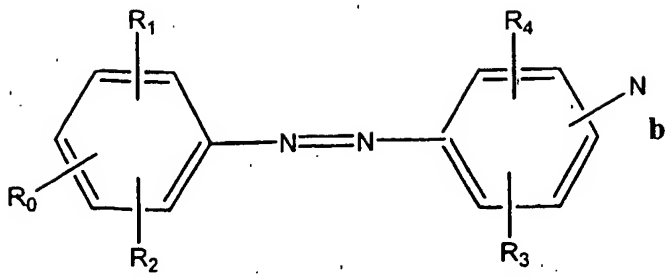
R_{10} and R_{11} independently are H, $-CN$, $-OR_{12}$, $-N(R_{12})_2$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated **a** symbolizes covalent attachment of the fluorophore to the linker; and

Q comprises a diazo moiety having the formula:



wherein covalent attachment to the linker is through the nitrogen atom designated as **b**.

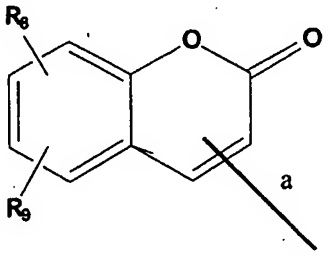
77. A method for hybridizing nucleic acids, comprising the steps of:

(a) providing a first nucleic acid and a second nucleic acid,

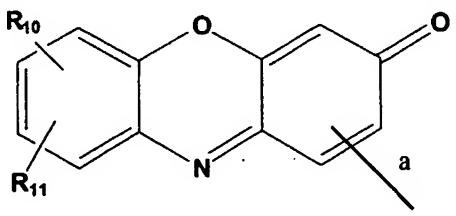
(b) incubating the nucleic acids under hybridization conditions, and

(c) identifying hybridized nucleic acids;

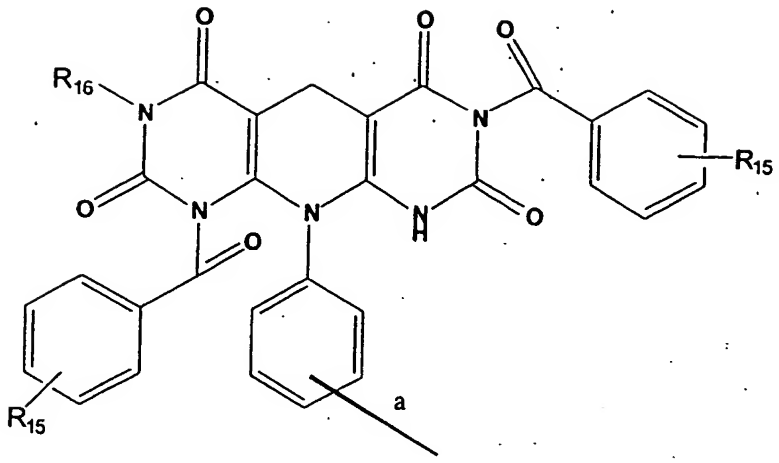
wherein at least one of the nucleic acids comprises a **FL-ODN-Q-MGB** conjugate where ODN is a nucleic acid or modified nucleic acid, MGB is minor groove binder moiety covalently attached to the ODN moiety or to the quencher moiety through a linker having the length of 0 to approximately 30 atoms, **Q** is a quencher moiety covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, and **FL** is a fluorophore moiety covalently attached to the nucleic acid through a linker having the length of 0 to approximately 30 atoms,, and the fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or $-CN$; $-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$, $-NHR_{nn}$, $-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

R_{10} and R_{11} independently are H, $-CN$, $-OR_{12}$, $-N(R_{12})_2$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated **a** symbolizes covalent attachment of the fluorophore to the linker.

78. A method for hybridizing nucleic acids, comprising the steps of:

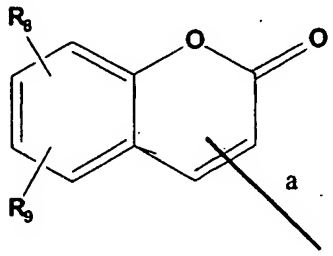
(a) providing a first nucleic acid and a second nucleic acid,

(b) incubating the nucleic acids under hybridization conditions, and

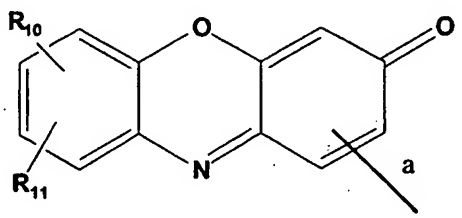
(c) identifying hybridized nucleic acids;

wherein at least one of the nucleic acids comprises a **FL-ODN-Q** conjugate where ODN is a nucleic acid or modified nucleic acid, **Q** is a quencher moiety covalently attached to the ODN through a linker having the length of 0 to approximately 30 atoms, and **FL** is a fluorophore moiety covalently attached to the nucleic acid through a linker having the length of 0

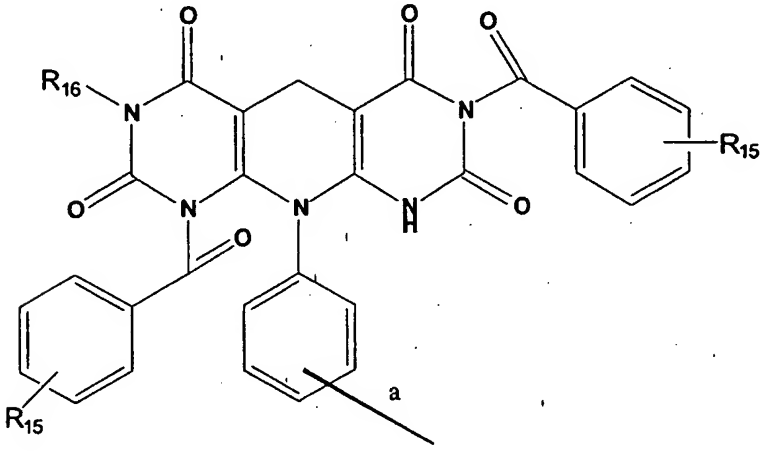
to approximately 30 atoms,, and the fluorophore moiety having the structure selected from the group designated FL-1, FL-2 and FL-3,



FL-1



FL-2



FL-3

wherein R_8 and R_9 independently are H, halogen, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, or –CN;$-OR_{nn}$, $-SR_{nn}$, $-OR_{nn}$,$-NHR_{nn}$,$-N[R_{nn}]_2$ where R_{nn} is independently H, an alkyl group of 1 to 10 carbons or an alkanoyl group of 1 to 10 carbons;

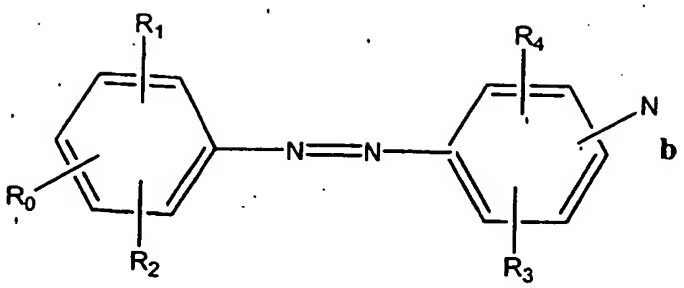
R_{10} and R_{11} independently are H, –CN , $-OR_{12}$, $-N(R_{12})_2$, halogen,-$O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, $-SO_3$, $-C(=O)NH_2$, $-N[(CH_2)_nCH_3]_2$ where n= 0 to 5, or R_{12} is alkyl of 1 to 10 carbons alkanoyl of 1 to 10 carbons,;

R_{15} is H or alkyl of 1 to 10 carbons;

R_{16} is alkyl of 1 to 10 carbons, and

the valence bond designated a symbolizes covalent attachment of the fluorophore to the linker; and

Q comprises a diazo moiety having the formula:



wherein covalent attachment to the linker is through the nitrogen atom designated as **b**.

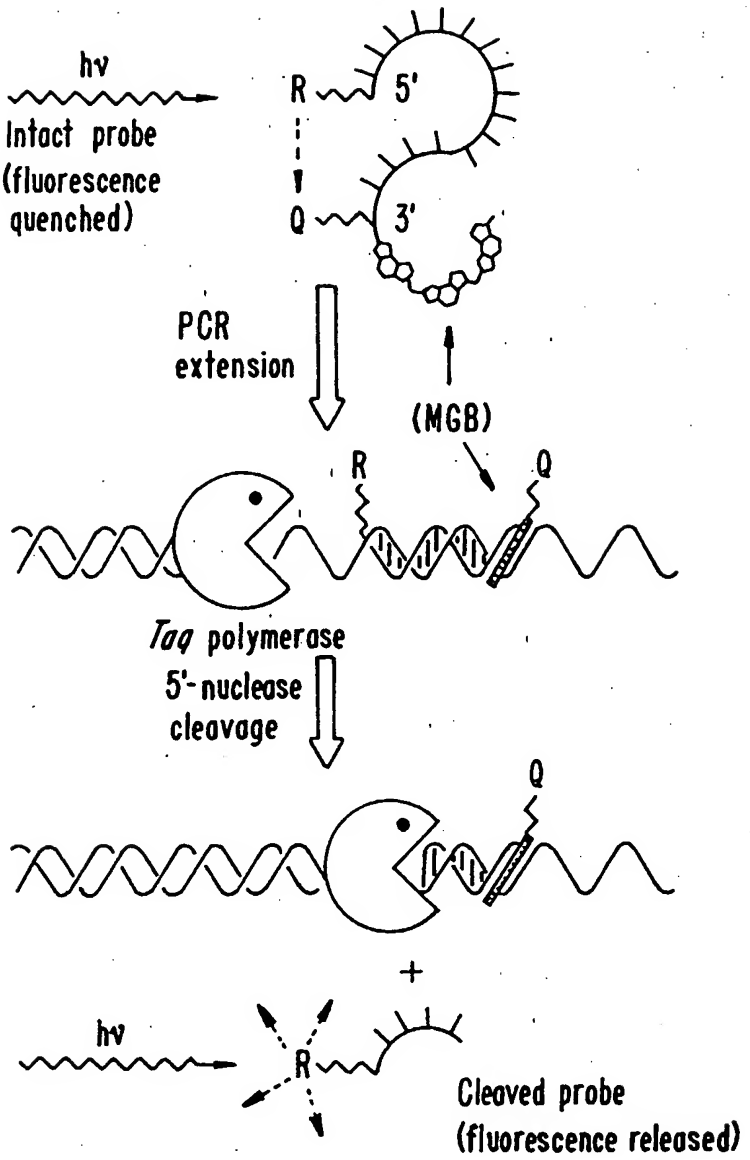

hv
Intact probe
(fluorescence
quenched)
R
5'
Q
3'
PCR
extension
(MGB)
R
Q
Taq polymerase
5'-nuclease
cleavage
Q
+
hv
R
Cleaved probe
(fluorescence released)

FIG. 1.

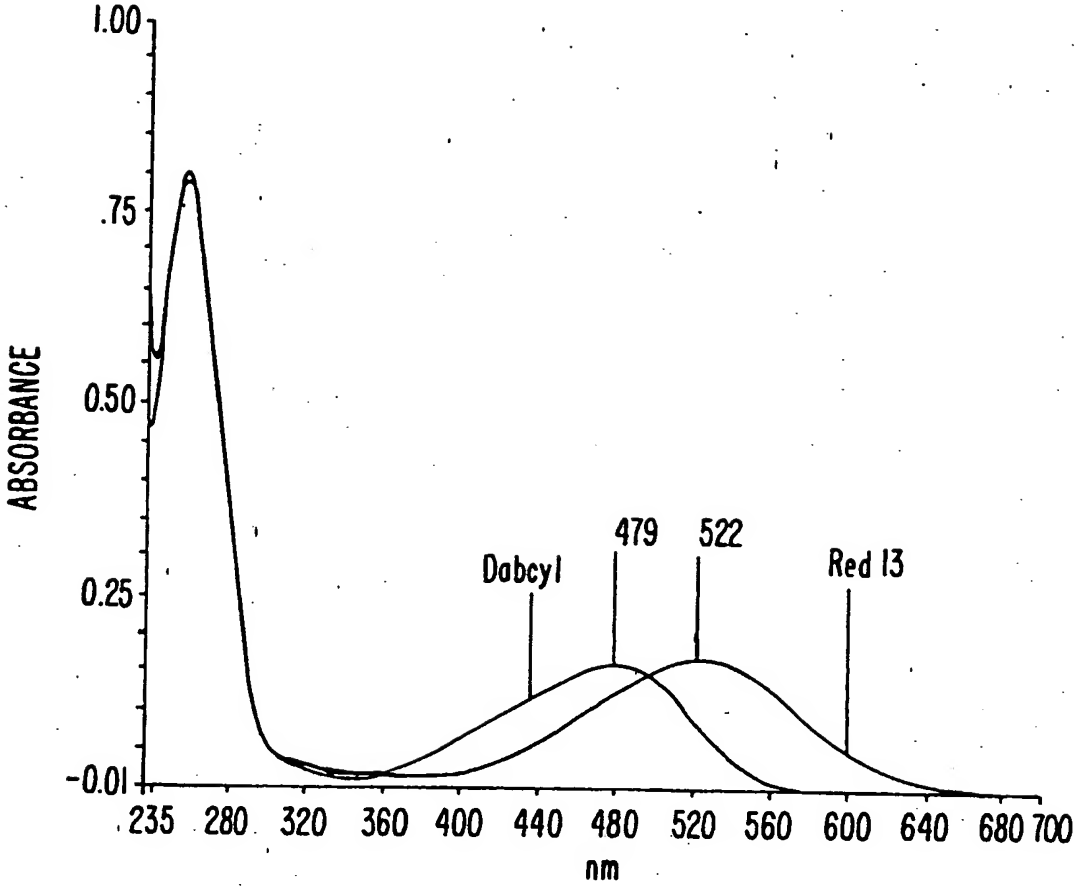

1.00
.75
0.50
0.25
-0.01
ABSORBANCE
Dabcyl
479
522
Red 13
235 280 320 360 400 440 480 520 560 600 640 680 700
nm

FIG. 2.

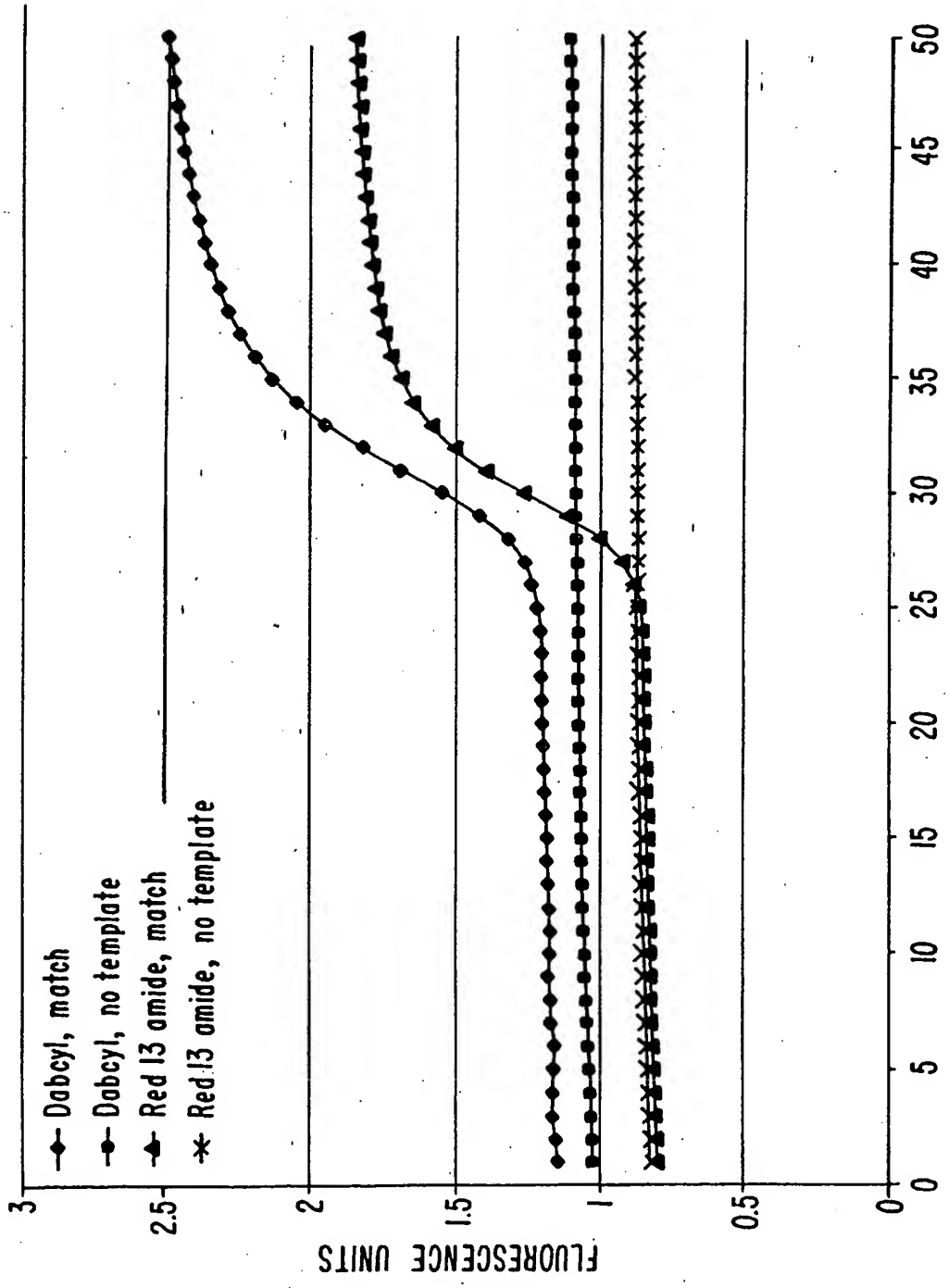

Dabcyl, match
Dabcyl, no template
Red 13 amide, match
Red 13 amide, no template
FLUORESCENCE UNITS
CYCLE #
0
0.5
1
1.5
2
2.5
3
0
5
10
15
20
25
30
35
40
45
50

FIG. 3.

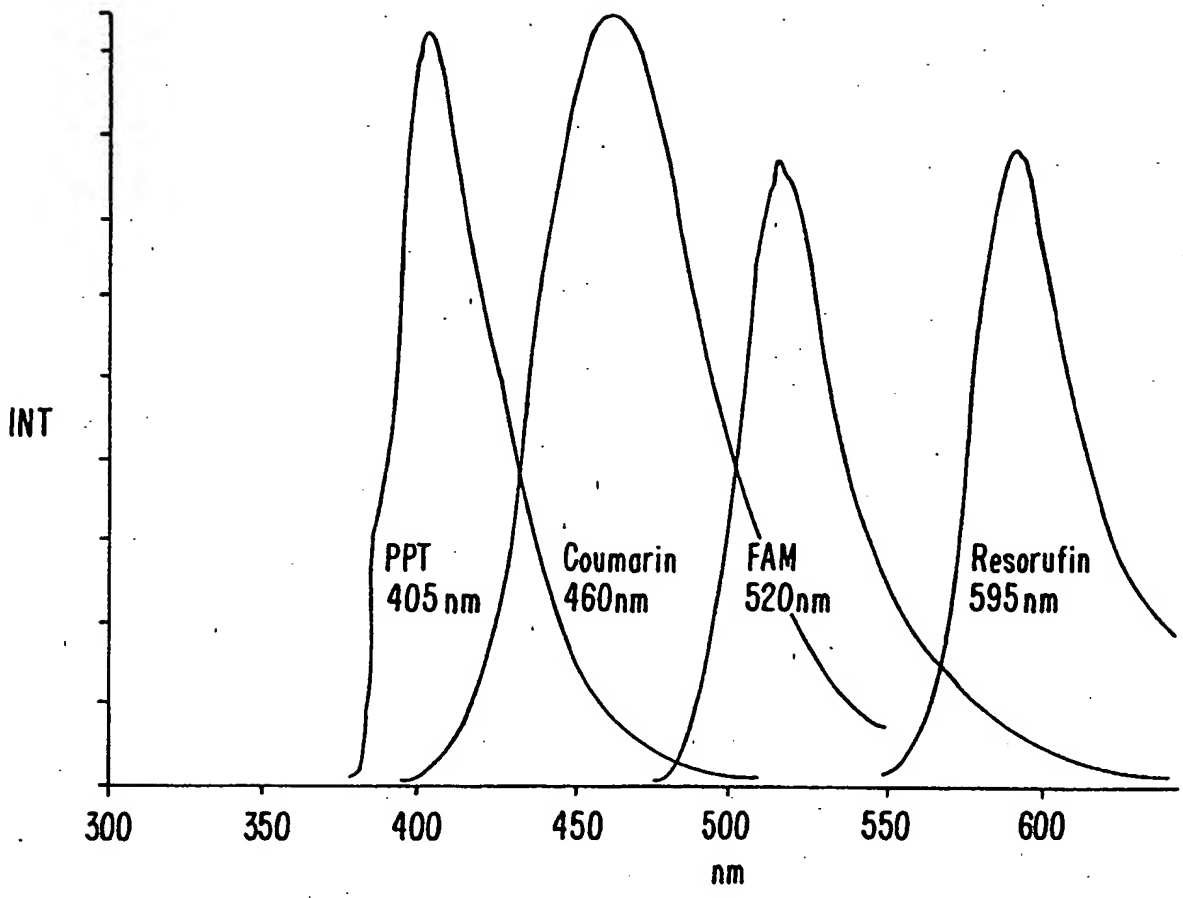

INT
PPT
405 nm
Coumarin
460nm
FAM
520nm
Resorufin
595nm
300
350
400
450
500
550
600
nm

FIG. 4.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US00/33333

(22) International Filing Date: 8 December 2000 (08.12.2000)

(25) Filing Language: English

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(30) Priority Data:
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(71) Applicant: EPOCH BIOSCIENCES, INC. [US/US]; 21720-23rd Drive, SE, #150, Bothell, WA 98021 (US).

(72) Inventors: REED, Michael, W.; 3575 NE 180th Street, Seattle, WA 98155 (US). LUKHTANOV, Eugeny, Alexander; 817 205th Street SE, Bothell, WA 98012 (US). GALL, Alexander, A.; 19701 10th Drive SE, Bothell, WA 98012 (US). DEMPCY, Robert, O.; 11421 NE 115th Court, Kirkland, WA 98033 (US).

(74) Agents: KEZER, William, B. et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, 8th Floor, San Francisco, CA 94111-3834 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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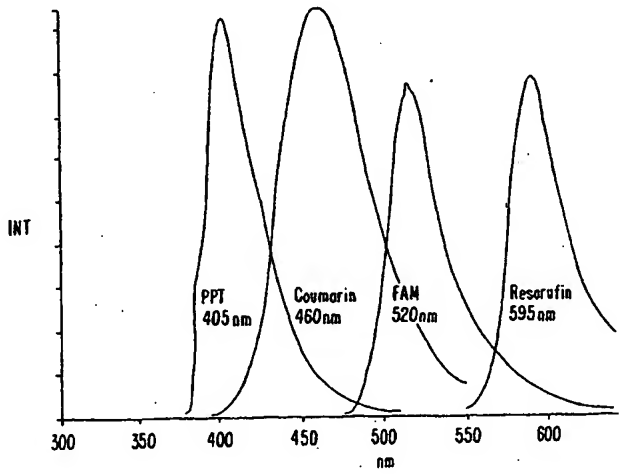
Published:
— *with international search report*

(88) Date of publication of the international search report:
24 January 2002

[Continued on next page]

(54) Title: FLUORESCENT QUENCHING DETECTION REAGENTS AND METHODS



(57) Abstract: Oligonucleotide-fluorophore-quencher conjugates wherein the fluorophore moiety has emission wavelengths in the range of about (300) to about (800) nm, and or where the quencher includes a substituted 4-(phenyldiazenyl)phenylamine structure provide improved signal to noise ratios and other advantageous characteristics in hybridization and related assays. The oligonucleotide-fluorophore-quencher conjugates can be synthesized by utilizing novel phosphoramidite reagents that incorporate the quencher moiety based on the substituted 4-(phenyldiazenyl)phenylamine structure, and or novel phosphoramidite reagents that incorporate a fluorophore moiety based on the substituted coumarin, substituted 7-hydroxy-3H-phenoxazin-3-one, or substituted 5,10-dihydro-10-[phenyl]pyrido[2,3-d:6,5-d']dipyrimidine-2,4,6,8-(1H, 3H, 7H, 9H, 10H)-tetrone structure. Oligonucleotide-fluorophore-quencher-minor groove binder conjugates including a pyrrolo[4,5-e]indolin-7-yl}carbonyl)pyrrolo[4,5-e]indolin-7-yl]carbonyl}pyrrolo[4,5-e]indoline-7-carboxylate (DPI_3) moiety as the minor groove binder and the substituted 4-(phenyldiazenyl)phenylamine moiety as the quencher, were synthesized and have substantially improved hybridization and signal to noise ratio properties.

WO 01/42505 A3

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 00/33333

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H21/00 C07C245/08 C07F9/24 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07H C07C C07F C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 39008 A (KRAMER FRED R ;TYAGI SANJAY (US); NEW YORK HEALTH RES INST (US)) 23 October 1997 (1997-10-23) claims figure 5 ---	1,22,23, 50,51,57
X	ASANUMA H ET AL: "Photo-Responsive Oligonucleotides Carrying Azobenzene in the Side-Chains" TETRAHEDRON LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 39, no. 49, 3 December 1998 (1998-12-03), pages 9015-9018, XP004140991 ISSN: 0040-4039 scheme 1 --- -/--	6

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Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2001	09. 08. 01
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer de Nooy, A

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 00/33333

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X'	US 5 304 645 A (KLEIN CHRISTIAN ET AL) 19 April 1994 (1994-04-19) cited in the application column 1 -column 4 column 5, line 16 - line 24	50,53
X	"'98 Catalogue" 1998 , AMERSHAM PHARMACIA BIOTECH , 'S-HERTOGENBOSCH, NETHERLANDS XP002172887 page 127, FluoroBlue	50,51
A	LEE L G ET AL: "ALLELIC DISCRIMINATION BZ NICK-TRANSLATION PCR WITH FLUOROGENIC PROBES" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 21, no. 16, 11 August 1993 (1993-08-11), pages 3761-3766, XP000470188 ISSN: 0305-1048 cited in the application page 3761 -page 3762	1,57
A	US 5 952 202 A (AOYAGI KAZUKO ET AL) 14 September 1999 (1999-09-14) the whole document	1,57
A	NAZARENKO ET AL: "A CLOSED TUBE FORMAT FOR AMPLIFICATION AND DETECTION OF DNA BASED ENERGY TRANSFER" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 25, no. 12, 1997, pages 2516-2521, XP002094959 ISSN: 0305-1048 the whole document	1,57
A	SOKOL D L ET AL: "Real time detection of DNA.RNA hybridization in living cells" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 95, no. 20, September 1998 (1998-09), pages 11538-11543, XP002123471 ISSN: 0027-8424 the whole document	1,57
A	US 5 801 155 A (GAMPER HOWARD B ET AL) 1 September 1998 (1998-09-01) claims	15,16

INTERNATIONAL SEARCH REPORT

International Application No
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2001	09. 08. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	de Nooy, A

INTERNATIONAL SEARCH REPORT

Int. Jonal Application No
PCT/US 00/33333

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	LEE L G ET AL: "ALLELIC DISCRIMINATION BZ NICK-TRANSLATION PCR WITH FLUOROGENIC PROBES" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 21, no. 16, 11 August 1993 (1993-08-11), pages 3761-3766, XP000470188 ISSN: 0305-1048 cited in the application page 3761 -page 3762	1,57
A	US 5 952 202 A (AOYAGI KAZUKO ET AL) 14 September 1999 (1999-09-14) the whole document	1,57
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A	US 5 801 155 A (GAMPER HOWARD B ET AL) 1 September 1998 (1998-09-01) claims	15,16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/33333

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-21, 22-28 (in part), 29 (in full), 30-33 (in part), 41-42 (in full), 57-58 (in full), 59-65 (in part), 66-67 (in full), 68-74 (in part), 75 (in full), 76-78 (in part)

A quencher moiety containing derivative where the quencher has the structure of the quencher moiety of claim 1.

2. Claims: 22-28 (in part), 30-33 (in part), 34-40 (in full), 43-56 (in full), 59-65 (in part), 68-74 (in part), 76-78 (in part)

A fluorophore containing derivative where the fluorophore has one of the structures of claim 22 and which is not falling within one of the previously described subjects.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. tional Application No
PCT/US 00/33333

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